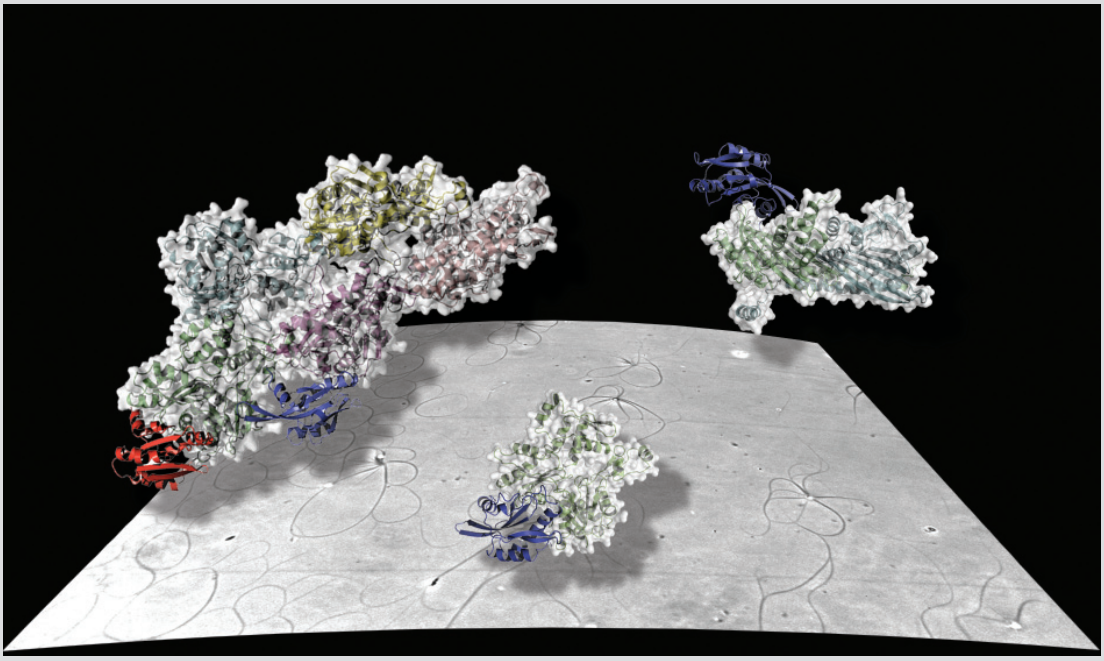

Structural Basis of Cytoskeletal Regulation by Twinfilin

VILLE O. PAAVILAINEN

Institute of Biotechnology and
Department of Biological and Environmental Sciences
Division of Genetics
Faculty of Biosciences
and
Helsinki Graduate School in Biotechnology and Molecular Biology
University of Helsinki

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Ville Paavilainen

Institute of Biotechnology,
Department of Biological and Environmental Sciences,
Division of Genetics,
University of Helsinki

Helsinki Graduate School in Biotechnology and Molecular Biology

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Supervised by:
Professor Pekka Lappalainen
Institute of Biotechnology
University of Helsinki, Finland

Reviewed by:

Docent Sarah Butcher
Institute of Biotechnology
University of Helsinki, Finland

and

Professor Jari Ylänne
Department of Biological and
Enviromental Science
University of Jyväskylä, Finland

Opponent:

Associate Professor Roberto Dominguez
University of Pennsylvania,
School of Medicine,
U.S.A.

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This thesis is based on the following original articles, which are referred to in the text by their roman numerals, and on unpublished results presented in the text.

I. Ojala P.J., **Paavilainen V.O.**, Vartiainen M.K., Tuma R., Weeds A.G., Lappalainen P. (2002). The two ADF-H domains of twinfilin play functionally distinct roles in interactions with actin monomers. *Molecular Biology of the Cell* 13 (11): 3811-3821.

II. **Paavilainen V.O.**, Merckel M.C., Falck S., Ojala P.J., Pohl E., Wilmanns M., Lappalainen P. (2002). Structural conservation between the actin monomer-binding sites of ADF/cofilin and twinfilin. *Journal of Biological Chemistry* 277 (45): 43089-43095.

III. Falck S., **Paavilainen V.O.**, Wear M.A., Grossmann J.G., Cooper J.A., Lappalainen P. (2004). Biological role and structural mechanism of twinfilin-capping protein interaction. *EMBO Journal* 23 (15): 3010-3019.

IV. **Paavilainen V.O.***, Hellman M.*, Helfer E., Bovellan M., Annila A., Carlier M.-F., Permi P., Lappalainen P. (2007): Structural basis and evolutionary origin of actin filament capping by twinfilin. *Proceedings of the National Academy of Sciences (in press).*

*) These authors contributed equally to this work.

ABBREVIATIONS

Abp1	Actin binding protein 1
ADP	Adenosine diphosphate
ADF-H	Actin depolymerizing-factor –homology
Aip1	Actin interacting protein 1
Arp	Actin related protein
ATP	Adenosine triphosphate
CAP	Cyclase associated protein
CARMIL	Capping protein, Arp2/3, Myosin I Linker
CP	Heterodimeric capping protein
DAD	Diaphanous auto-regulatory domain
DID	Diaphanous inhibitory domain
F-actin	Filamentous actin
FH1	Formin homology 1 domain
FH2	Formin homology 2 domain
G-actin	Monomeric actin
NPF	Nucleation promoting factor
N-WASP	Neural WASP protein
PAGE	Polyacrylamide gel electrophoresis
$PI(4,5)P_2$	Phosphatidyl-inositol 4,5-diphosphate
$PI(3,4,5)P_3$	Phosphatidyl-inositol 3,4,5-triphosphate
SAD	Single wavelength anomalous diffraction
SAXS	Small-angle x-ray scattering
Tmod	Tropomodulin
Twf-C	C-terminal domain of twinfilin
Twf-N	N-terminal domain of twinfilin
VASP	Vasodilator-stimulated phosphoprotein
VCA	Verprolin, Central, Acidic
WASP	Wiscott-Aldrich syndrome protein
WAVE	WASP family verprolin homologous protein
WH1	WASP homology domain 1
WH2	WASP homology domain 2
WIP	WASP interacting protein
Å	Ångström ($=10^{-10}$m)

ABSTRACT

The actin cytoskeleton is required, in all eukaryotic organisms, for several key cellular functions such as cell motility, cytokinesis, and endocytosis. In cells, actin exists either in a monomeric state (G-actin) or in a filamentous form (F-actin). F-actin is the functional form, which can assemble into various structures and produce direct pushing forces that are required for different motile processes. The assembly of actin monomers into complicated three-dimensional structures is tightly regulated by a large number of actin regulating proteins.

One central actin regulating protein is twinfilin. Twinfilin consists of two actin depolymerizing-factor homology (ADF-H) domains, which are capable of binding actin, and is conserved from yeast to mammals. Previously it has been shown that twinfilin binds to and sequesters G-actin, and interacts with the heterodimeric capping protein. More recently it has been found that twinfilin also binds to the fast growing actin filament ends and prevents their growth. However, the cellular role of twinfilin and the molecular mechanisms of these interactions have remained unclear. In this study we characterized the molecular mechanisms behind the functions of twinfilin. We demonstrated that twinfilin forms a high-affinity complex with ADP-bound actin monomers (ADP-G-actin). Both ADF-H domains are capable of binding G-actin, but the C-terminal domain contains the high-affinity binding site. Our biochemical analyses identified twinfilin's C-terminal tail region as the interaction site for capping protein. Contrary to G-actin binding, both ADF-H domains of twinfilin are required for the actin filament barbed end capping activity. The C-terminal domain is structurally homologous to ADF/cofilin and binds to filament sides in a similar manner, providing the main affinity for F-actin during barbed end capping. The structure of the N-terminal domain is more distant from ADF/cofilin, and thus it can only associate with G-actin or the terminal actin monomer at the filament barbed end, where it regulates twinfilin's affinity for barbed ends. These data suggest that the mechanism of barbed end capping is similar for twinfilin and gelsolin family proteins. Taken together, these studies revealed how twinfilin interacts with G-actin, filament barbed ends, and capping protein, and also provide a model for how these activities evolved through a duplication of an ancient ADF/cofilin-like domain.

1. REVIEW OF THE LITERATURE

1.1. The cytoskeleton

All eukaryotic cells possess a cytoskeleton that is composed of filamentous protein assemblies. These protein assemblies play an important role during several key cellular processes such as cell motility, cell division, endocytosis, and morphogenetic movements. Three types of filamentous networks exist in eukaryotic cells: microtubules, intermediate filaments, and actin filaments.

The microtubule system consists of long filaments formed by two homologous proteins, α- and β-tubulin. They provide a platform for several intracellular transport events. During mitosis, the microtubule cytoskeleton is responsible for the correct segregation of sister chromatids. The microtubules are used as 'tracks' along which the motor proteins, kinesins and dyneins, can travel. The intermediate filament system consists of several different proteins and is involved in a wide variety of cellular processes, and also provides mechanical strength to, for example, epithelial and neuronal cells. Contrary to microtubules and intermediate filaments, the actin cytoskeleton consists of a single protein, actin (reviewed in Alberts Bruce et al., 1994).

1.2. Actin

Actin is one of the most highly conserved and abundant proteins found

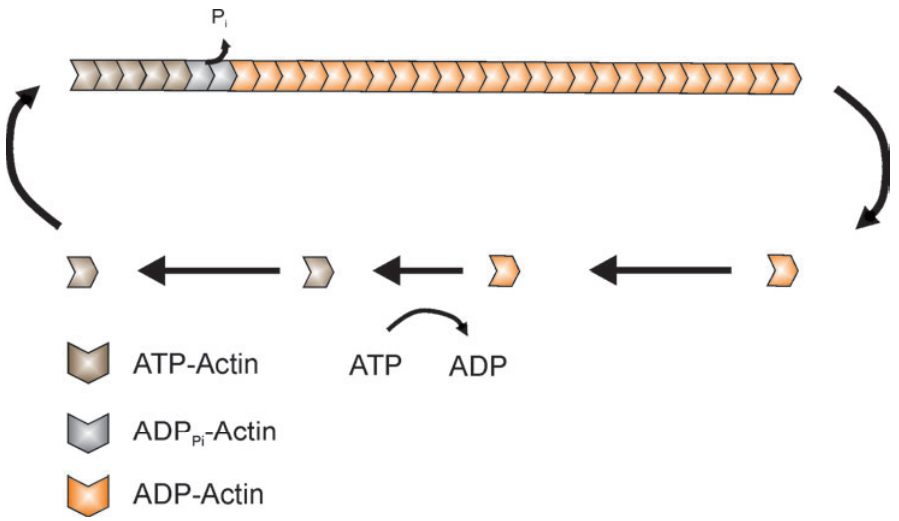
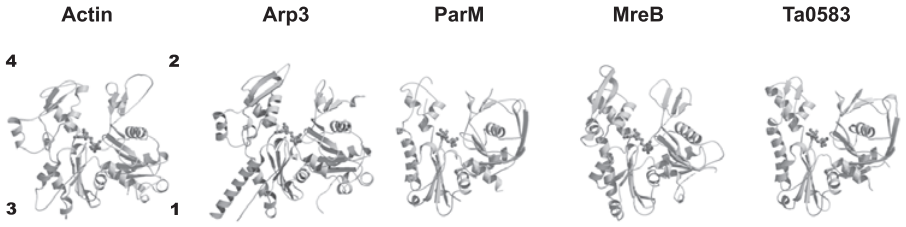


Fig 1. The treadmilling cycle of actin. Actin monomers in their ATP-bound state prefer assembly to the barbed end of the actin filament. After association, the γ-phospate of ATP is rapidly hydrolyzed, followed by relatively slow diffusion of the P_i -moiety out of the filament. After P_i-release, the ADP-bound actin subunits prefer to dissociate from the pointed end of the actin filament. After nucleotide exchange, the actin monomers are able to re-assemble to the barbed end. This cycle of actin polymerization and depolymerization is termed treadmilling, and in cells all of these steps are highly regulated by actin regulating proteins.

in all eukaryotes. Actin has an ability to polymerize into polarized filaments that are constantly turning over. The controlled polymerization/depolymerization cycle (Figure 1) forms the basis for the function of the actin cytoskeleton, which is vital for several key cellular events such as cell motility, cell division, and endocytosis. In multicellular organisms, the actin cytoskeleton is required for several morphogenetic processes, such as movement of neurites during development, remodeling of the nervous system, and chemotactic movements of for example fibroblasts during wound healing. All of these processes are dependent on cell motility and require coordinated assembly of actin filaments into specialized networks, which provide the driving force for several cellular functions (reviewed in Pollard et al., 2000).

Actin filaments are polar two-stranded structures that have a fast-growing barbed end (named because of the arrowhead pattern created when myosin binds to actin filaments) and a slow-growing pointed end (Craig et al., 1985). To generate directed actin polymerization in response to extracellular signals, the cell must be able to control actin polymerization both spatially and temporally via cellular signaling pathways. This control takes place on a timescale of seconds to minutes and is very rapid in comparison with for example protein synthesis (reviewed in Pollard et al., 2000).

The actin molecule consists of four subdomains, an ATP- or an ADP -molecule bound in a cleft between actin subdomains 2 and 4 (Figure 2), and either a Mg^{2+}- or a Ca^{2+}-ion associated with the nucleotide (Kabsch et al., 1990, Otterbein et al., 2001). The nucleotide- and Mg^{2+}/Ca^{2+} -status is important for the biochemical properties of actin. The favored state for filament assembly is Mg-ATP-actin. The filament ends have different on- and off-rates for actin monomers; the barbed ends preferably assemble actin monomers in their ATP-bound state, whereas the



	Actin	Arp3	ParM	MreB	Ta0583
RMSD (Å)	0.0	2.2	3.0	3.8	3.0
Z-Score	62.9	46.7	22.7	29.2	22.7
Seq Id. (%)	100	39	14	15	14

Fig 2. Comparison of actin with it's prokaryotic homologs. The monomer structures of actin, Arp3, ParM, MreB and Ta0583 (PDB codes 1atn, 1k8k, 1mwm, 1jcg and 2fsj, respectively) were aligned with the program DALI (Holm and Sander, 1998). The random mean square displacement values, Z-scores, sequence identities between the Cα-atoms of actin and the different actin homologs were obtained from the program DALI. Comparison of actin with the non-related tubulin homolog, gives an RMSD-value of 4.2, and a Z-score of 0.7. The protein is represented as ribbons, and nucleotide as ball-and-sticks. The actin subdomains are indicated in the first panel. The picture was created with the programs Molscript (Kraulis, 1991) and RASTER3D (Merrit et al., 1994).

pointed end prefers the disassembly of actin monomers in their ADP-bound state (Figure 1). Upon association with the barbed end, the ATP in actin is rapidly hydrolyzed and the majority of actin monomers in the filament are thus in the ADP-bound form. These chemical events are responsible for the so-called tread-milling effect of actin filaments, where the filaments effectively grow at the barbed ends and shrink at the pointed ends. In cells the assembly and disassembly of actin filaments, as well as their organization into distinct three-dimensional networks, are regulated by a large number of actin-binding/regulating proteins (reviewed in Sheterline et al., 1995). The different forms of actin enable the binding of a large number of actin-binding proteins to a specific form of actin.

1.3. Actin related proteins and prokaryotic actin homologs

The actin molecule is composed of a single domain that has four subdomains (Figure 2). Subdomains 1 and 3 form the 'barbed end' of the actin molecule, which is the interaction site for many actin binding proteins (McLaughlin et al., 1993; Schutt et al., 1993; Hertzog et al., 2004; Otterbein et al., 2002). Subdomains 2 and 4 define the 'pointed end'. The actin fold is functionally characterized by the ability to bind ATP in the presence of Mg^{2+} or Ca^{2+} (reviewed in Kabsch and Holmes, 1995).

In addition to 'conventional' actin, many proteins display significant sequence and structural homology to actin (Figure 2), forming the actin related protein (Arp) superfamily (reviewed in Schafer and Schroer, 1999). Ten classes of actin related proteins have been identified so far. Most of them bind ATP and it is possible that ATP hydrolysis is important for their function (Muller et al., 2005). Arp1 is a member of the 11-subunit dynactin complex, which functions in the transport of cargo and organelles on microtubules (Gill et al., 1991). In humans, this complex also includes Arp11 as well as monomeric actin and capping protein. So far Arp1 is the only member of the Arp family known to form filaments. Arps 2 and 3 are members of the seven-subunit Arp2/3 complex, where they serve to nucleate new actin filament ends (reviewed in Goley and Welch, 2006). Arps 4-9 are nuclear proteins which exist together with other proteins in large, >2 MDa chromatin remodeling complexes (reviewed in Blessing et al., 2004).

More recently, also bacterial homologs of actin have been identified (Figure 2). The bacterial protein FtsA was shown to display structural similarity to actin (van den Ent and Lowe, 2000). Despite the overall similarity, this protein has strikingly different subdomain organization and no evidence exists that it could polymerize. Another bacterial protein, called MreB, was shown to be important for the maintenance of cell shape in rod-like bacteria and in chromosome segregation during mitosis (Doi et al., 1988). Subsequently the atomic structure of MreB was solved and was shown to be very similar to actin. MreB also assembles into filaments *in vitro* and into a coiled filamentous structure near the plasma membrane in cells (van den Ent et al., 2001). Thus MreB was the first functional actin homolog identified from bacteria. Another protein, called ParM, was subsequently discovered as another bacterial actin homolog. ParM is involved in pushing newly replicated copies of a plasmid to opposite poles of the cell during plasmid segregation before cell division. ParM forms actin-like filaments *in vitro*, in a manner dependent on ATP hydrolysis, and the atomic structure of this protein has

shown that it is very similar to actin (van den Ent et al., 2002). Unlike actin filaments, ParM filaments display microtubule-like ATP-dependent, dynamic instability (Garner et al., 2004). More recently, a protein called Ta0583, was shown to be structurally similar to actin, to possess an ATPase activity, and to form sheet-like filamentous structures *in vitro* (Roeben et al., 2006). The *in vivo* function of Ta0583 still remains unknown. Also a homolog of eukaryotic tubulin, called FtsZ, has been characterized in bacteria (Lowe and Amos, 1998). However, unlike MreB or ParM, this protein polymerizes *in vitro* into different forms, none of which resemble the eukaryotic microtubules. Contrary to eukaryotic organisms, the tubulin homolog FtsZ, is required for bacterial cytokinesis, whereas the actin homolog MreB is implicated in chromosome segregation (reviewed in Margolin, 2005). It is clear that actin homologous proteins in prokaryotes have utilized the actin fold to regulate numerous aspects of cellular function.

1.4. Actin-binding/regulating proteins

1.4.1. Actin nucleating proteins

Three possibilities exist for generating new actin filament barbed ends to promote rapid actin filament growth: 1) existing filaments can be severed to create more barbed ends, 2) existing filaments can be uncapped, relieving the inhibition of barbed end growth, and 3) new filaments can be nucleated *de novo* (reviewed in Pollard and Borisy, 2003). Cells utilize several mechanisms to promote coordinated actin filament assembly. In this chapter the general mechanisms of actin nucleation and the three known actin nucleators will be introduced.

1.4.1.1. The Arp2/3 complex

The Arp2/3 complex is a large (MW ~220 kDa) seven subunit protein complex that is conserved among all eukaryotes. It consists of five novel subunits and two actin related proteins (Machesky et al., 1994). This complex is believed to be responsible for generation of the so-called dendritic actin filament network (Figure 3), responsible for force production near the plasma membrane in mammalian cells (Welch et al., 1997; Mullins et al., 1998). The Arp2/3 complex binds to the sides of pre-existing actin filaments and catalyzes the formation of new actin filaments from the side of the 'mother filament', creating a characteristic 70 degree angle between the mother and daughter filaments. The structure of the inactive complex, solved by x-ray crystallography (Robinson et al., 2001; Nolen et al., 2004), together with electron cryomicroscopy analysis of the activated Arp2/3 complex (Volkmann et al., 2001) suggested that the two actin related proteins in the complex would act as the first subunits of the daughter filament.

The Arp2/3 complex alone is very inefficient in promoting the nucleation of new actin filaments, and in cells its actin nucleating activity is tightly regulated by several activating proteins (Figure 3) (reviewed in Goley and Welch, 2006). These activating proteins can be divided into class I and class II nucleation promoting factors (NPFs). Class I NPFs contain a so-called VCA-domain (described in chapter 1.4.4.3) that binds to G-actin and the Arp2/3 complex, and this group includes proteins such as Wiscott-Aldrich syndrome protein (WASP), Neural-WASP protein (N-WASP), WASP family verprolin homologous protein (SCAR/WAVE), Myosin-I and Capping

protein, Arp2/3, Myosin I Linker protein (CARMIL). Also the bacterial proteins ActA and RickA belong to class I NPFs. These proteins bind the Arp2/3 complex, mediate an activating conformational change of the complex, and recruit an actin monomer. After nucleation of the daughter filament, class I NPFs dissociate from the Arp2/3 complex. Class II NPFs contain an Arp2/3 binding acidic region but lack a G-actin binding region. Instead these proteins bind F-actin, which is required for Arp2/3 activation. Class II NPFs include actin binding protein 1 (Abp1), Pan1, and cortactin (reviewed in Goley and Welch, 2006). The Arp2/3 activating mechanism of class II NPFs is not clear and instead of acting as nucleation promoting factors in cells their role might be to stabilize actin filament branches catalyzed by class I NPFs (Weaver et al., 2001)

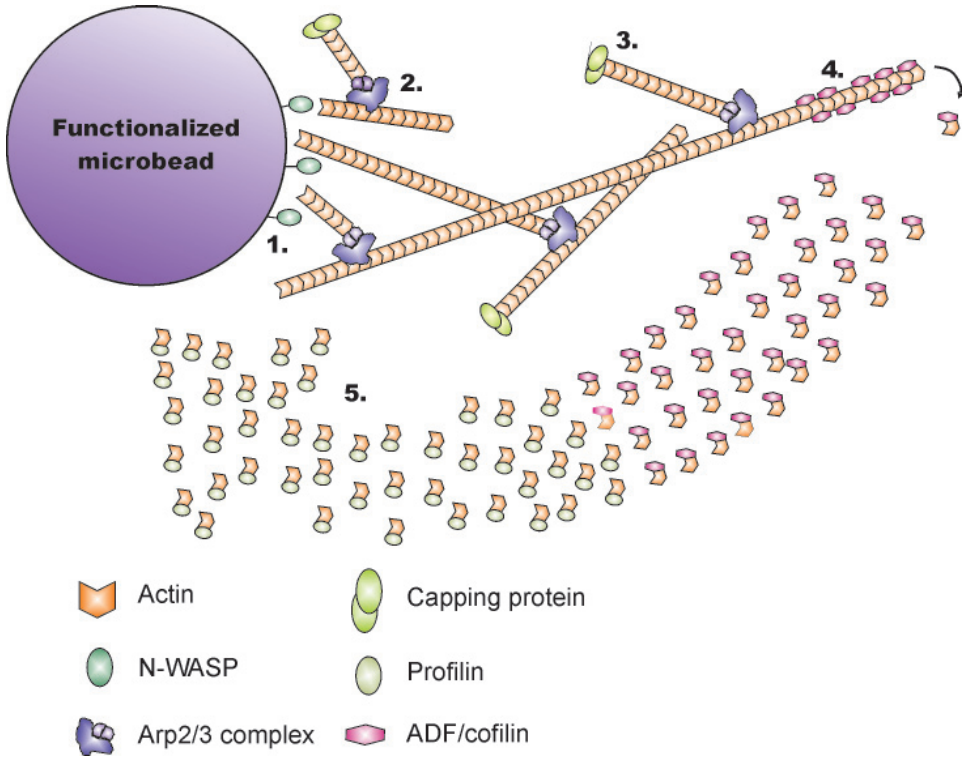


Fig 3. A schematic drawing describing the 'dendritic nucleation model' (Pollard et al., 2000). In the model **1)** proteins of the WASP/WAVE family activate the Arp2/3 complex to **2)** nucleate new filaments from the sides of pre-existing ones. After the new filament has grown for a while it **3)** becomes capped by capping proteins, and therefore can not grow further. After the filaments age, **4)** ADF/cofilin proteins promote their depolymerization at the filament pointed ends. **5)** The large unpolymerized actin monomer pool is maintained by a set of actin monomer sequestering proteins. Profilin catalyzes the exchange of ADP to ATP in actin monomers, thus enabling further rounds of polymerization at a fast rate. The purple circle represents a functionalized microbead, with N-WASP protein covalently attached. In the context of the cell, the actin machinery is located at the plasma membrane, thus enabling pushing forces on the membrane. The same proteins are required in a biomimetic bead motility assay, which was used during this work.

The newly formed actin filament branches release the daughter filament a few minutes after formation *in vitro* (Le Clainche et al., 2003). Both Arp2 and Arp3 bind ATP (Dayel et al., 2001). ATP binding to Arp2 is enhanced by activation of Arp2/3 and is required for filament branching (K_D = 40 μM in the absence and 0.1 μM in the presence of WASP activating region) (Le Clainche et al., 2003). However, ATP binding of Arp3 is not affected by WASP activation. ATP binding (or hydrolysis) by Arp2 and Arp3 could bring these two subunits together to form the active structure of the Arp2/3 complex. ATP hydrolysis has been observed for Arp2 *in vitro*, but not for Arp3 (Le Clainche et al., 2003; Dayel et al., 2001). In yeast cells, a mutation inhibiting Arp2 ATP hydrolysis caused a severe defect in endocytotic activity reflecting the *in vivo* importance of ATP hydrolysis for Arp2/3-mediated force production (Martin et al., 2006). Interestingly, a comparable defect was also observed with a similar mutation in Arp3, hinting that also this subunit could participate in ATP hydrolysis (Martin et al., 2006). The same Arp2 and Arp3 mutations were shown to stabilize the branches of the daughter filaments *in vitro*. It was also shown that Las17 (yeast WASP protein) caused an increase in the rate of branch dissociation. However since the Arp2/3 ATP hydrolysis was much slower *in vitro* than the life-time of yeast actin patches, it was proposed that additional factors, like cofilin, would be involved in regulating the debranching process in cells (Martin et al., 2006). In conclusion, debranching appears to be a novel feature of Arp2/3, important for actin mediated force production at least during endocytosis. However, the role of ATP hydrolysis in Arp2/3 nucleation and debranching and the recycling of the complex remains unclear.

1.4.1.2. Formins

Formins are a large family of homodimeric multidomain proteins capable of nucleating new actin filaments. Formins are found in organisms ranging from yeast to mammals and most organisms have multiple formin proteins: two exist in *Saccharomyces cerevisiae*, six in *Drosophila*, and 15 in mammals. Contrary to the Arp2/3 complex, formins promote the growth of non-branched, unidirectional actin filament structures implicated in a variety of cellular functions (reviewed in Kovar, 2006).

All formin proteins posses a variable length formin homology 1 domain (FH1) and a neighboring, highly conserved, formin homology 2 domain (FH2). These are surrounded by additional regulatory domains, depending on the formin protein. The conserved FH2 domain is the 'signature' domain of different formins, and based on this domain formins can be divided into seven phylogenetic classes: Dia (diaphanous), DAAM (dishevelled-associated activator of morphogenesis), FRL (formin related gene in leukocytes), FHOD (formin-homology-domain containing protein), INF (inverted formin), FMN (formin) and delphilin (reviewed in Higgs, 2005).

The ~400 residue FH2 domain homodimerizes to form a flexible but stable 'donut' -like structure, which binds to the filament barbed ends with nanomolar affinity. The FH2-dimer binds to barbed ends processively, i.e. the protein remains bound at the barbed end while still allowing filament elongation to proceed. The structure of the FH2 domain alone and with actin (Xu et al., 2004; Otomo et al., 2005) suggested that the homodimeric domain would interact with two actin subunits at the barbed end, and that during

the processive binding, one of these interactions would be disrupted at a time. It has been proposed that the FH1-FH2 -construct accelerates the ATP hydrolysis on profilin-bound actin monomers, and that this drives the processive polymerization reaction (Romero et al., 2004). However, another study suggested that actin ATP hydrolysis is not required for the processivity (Kovar et al., 2006). The FH1 domain contains proline-rich stretches of varying length that facilitate a low-affinity interaction with profilin (reviewed in Higgs, 2005). This interaction is required for the ability of the FH1-FH2 -construct to promote barbed end assembly from profilin-actin complexes. Three possibilities exist for how the FH1-profilin interaction might contribute to the growth of specific actin filament arrays: 1) binding of FH1 to profilin-actin might increase the local concentration of actin monomers near the fast growing filament ends, 2) the controlled delivery of actin monomers by profilin might position them at the barbed end in an orientation that would promote their assembly to the actin filament and 3) the binding of profilin-actin might modulate formin's processivity (reviewed in Higgs, 2005). It has been proposed that formins exist either in a 'closed' state, still able to bind profilin-actin, but not allowing filament assembly at the barbed end, or in an 'open' state, which would allow both profilin-actin binding and association at the barbed end (Otomo et al., 2005). All formin proteins studied so far enhance actin filament polymerization at the barbed ends with profilin-actin, but with different binding parameters (reviewed in Kovar, 2006).

Different formins appear to be involved in the generation of distinct actin structures in cells and this may explain why different formins have specific regulatory regions outside the FH1-FH2 domains. In the best characterized formin, mouse diaphanous 1 (mDia1), the FH1-FH2 -region is autoinhibited by a regulatory interaction of it's diaphanous auto-regulatory domain (DAD) and diaphanous inhibitory domain (DID). Binding of Rho to a region near the DID domain relieves this inhibition and enables the FH1-FH2 -region to nucleate new actin filaments and promote their growth at the barbed ends (reviewed in Higgs, 2005).

1.4.1.3. Spire

Spire is the most recently discovered actin filament nucleating protein. Unlike Arp2/3 and formins, spire is only found in metazoan organisms. Spire was originally identified in *Drosophila* as a protein harboring multiple copies of the WASP homology domain 2 (WH2) domain. Over-expression of this protein in cells induced the formation of actin filament structures that did not co-localize with the Arp2/3 complex (Quinlan et al., 2005).

The isolated four-WH2-domain repeat of Spire was found to display an actin filament nucleating activity. Like actin filaments nucleated by formins, also Spire-nucleated filaments were straight. However, in contrast to formins, Spire remains bound to filament pointed end after nucleation, allowing barbed end growth, while preventing pointed end disassembly. It was proposed that the most C-terminal WH2 domain would initially bind a single actin monomer, after which the other three WH2 domains would recruit three more actin monomers, thus forming a short single-stranded actin polymer. This strand would then serve as a template for the assembly of the final two-stranded actin filament (Quinlan et al., 2005).

More recently it was shown that the Spire locus encodes three different proteins with different biochemical properties: SpireA, SpireC and SpireD, and it was SpireD which was previously shown to nucleate new actin filaments. It was also shown that, together with a formin protein called Cappuccino, some Spire proteins possess an F-actin/microtubule cross-linking activity. SpireC can cross-link F-actin and microtubules, but does not contain the four WH2 domains, which are required for actin filament nucleation. SpireD binds to Cappuccino, does not cross-link F-actin with microtubules, but instead inhibits the cross-linking activity of spireC. Thus it seems that spireC and spireD, together with Cappuccino, are regulating different aspects of cytoskeletal coordination involving the actin and microtubule cytoskeletons (Rosales-Nieves et al., 2006).

1.4.2. Actin cross-linking proteins.

Fundamental cellular processes, like cell motility, are dependent on the formation of complex three-dimensional actin filament networks. Formation of these filament networks is promoted by a wide variety of actin cross-linking proteins. In addition, these proteins function in cross-linking specific cell surface receptors and adhesion molecules with the underlying actin cytoskeleton. The actin cross-linking proteins include for example spectrin, fimbrin, α-actinin, and filamin. All of the proteins have a similar domain organization with two actin binding domains at both ends of a long rod-like dimer. Also some of these proteins act as tetramers. The best characterized member of this protein superfamily is α-actinin, which is introduced in more detail below.

1.4.2.1. α-actinin

α-actinin is a ubiquitous actin filament cross-linking protein that belongs to the spectrin superfamily (reviewed in Djinovic-Carugo et al., 2002). A-actinin is found in most eukaryotes like human, mouse, *Drosophila*, and *Caenorhabditis elegans*. Also fission yeast (*Saccharomyces pombe*) appears to have an α-actinin gene, but bakers yeast (*Saccharomyces cerevisiae*) or plants do not (Virel and Backman, 2004). α-actinin is an elongated homodimer with a molecular weight of 93-103 kDa. It consists of an N-terminal actin binding region, formed by two calponin homology (CH) domains, and a C-terminal calmodulin-like domain, formed by two EF-hand motifs. Next to the actin binding region are four spectrin repeats, which form the dimer-forming central rod region of α-actinin. In other cross-linking proteins such as filamin, the dimeric linker region consists of a set of Ig domains instead of spectrin repeats (Pudas et al., 2005).

Four α-actinin isoforms are found in mammals and probably in all vertebrates. α-actinins 2 and 3 function in striated muscle cells and cross-link actin filaments at the Z-disks of sarcomeres. In non-muscle cells, α-actinin 1 is primarily found in actin stress fibres, focal adhesions, and the leading edge, whereas α-actinin 4 localizes to certain membrane ruffles and appears to play a role in endocytosis and tumor cell motility (reviewed in Otey and Carpen, 2004).

α-actinins cross-link actin filaments to bundles and networks. They also bind several transmembrane proteins, such as integrins, cadherins, and intercellular adhesion molecules (ICAM) and thereby connect the actin cytoskeleton to the plasma membrane. Additionally this interaction might serve to cluster signaling

molecules and modulate the activity of some cell surface receptor molecules such as L-selectin. The cytoplasmic binding partners of α-actinin include for example vinculin, zyxin, extracellular signal-regulated kinase 1/2 (Erk1/2), mitogen activated protein/extracellular signal-regulated kinase 1 (MEKK1), protein kinase N (PKN), and phosphatidylinositol-3-kinase (PI3-K) to name but a few. The huge amount of binding partners indicates that an important function of α-actinin in cells is to act as a binding platform for other proteins (reviewed in Brakebusch and Fassler, 2003).

Other binding partners of α-actinin include phosphatidyl-inositol 4,5-diphosphate [$PI(4,5)P_2$] and phosphatidyl-inositol 3,4,5-triphosphate [$PI(3,4,5)P_3$], which regulate the binding of α-actinin to actin and other proteins. α-actinin is also regulated by tyrosine phosphorylation by the integrin-activated kinase focal adhesion kinase (FAK), which decreases α-actinin's affinity for actin. Dephosphorylation of α-actinin is mediated by tyrosine phosphatases SHP-1 and PTP1B (Lin et al., 2004; Zhang et al., 2006). A further level of α-actinin regulation is exerted by calcium binding to the EF-hand motifs, which also causes a reduction in actin affinity in the non-muscle isoforms. Null mutations of α-actinin in *Drosophila* result in lethality caused by muscular defects. Also photo-inactivation of α-actinin in mammalian cells results in loss of integrity of the focal adhesion-actin cytoskeleton linkage. However, depletion of α-actinin does not seem to affect focal adhesion integrity (Rajfur et al., 2001).

1.4.3. Actin filament capping proteins

Actin filament capping proteins bind to the ends of actin filaments and prevent the addition and loss of actin subunits to/from filament ends (Figure 3). These proteins antagonize the net growth of filaments, but are vital for actin based motility for two reasons: 1) capping limits how long the filaments can grow, thus resulting in a large amount of shorter, less flexible filaments that are better suited for generating pushing forces e.g. on the leading edge of mammalian cells, and 2) active capping effectively controls the localization of fast growing actin filament ends, and prevents the unwanted growth of actin filaments in other places of the cell (reviewed in Pollard and Borisy, 2003).

1.4.3.1. Gelsolins

Gelsolin is a ubiquitous barbed end capping protein, which is found in higher eukaryotes, but not in yeast. However, gelsolin proteins are found in plants, which points to the existence of an ancient gelsolin protein before the divergence of plants and animals. Gelsolin is a large protein (~83 kDa), composed of six homologous compact domains (125-150 amino acids each). The six gelsolin domains (G1-G6) are connected by linker regions of varying length. In addition to cytoplasmic gelsolin, a secreted form exists in the plasma of mammals that carries a 25-residue C-terminal extension. This form is required to prevent actin polymerization in blood circulation (reviewed in McGough et al., 2003). Yet other members of the family exist with only three gelsolin domains. Some of these proteins e.g. severin possess filament severing and capping activities. Additional members of the superfamily include villin, which has a C-terminal F-actin binding domain, and CapG, which can cap filament barbed ends but does not possess a severing activity (reviewed in Archer et al., 2005).

Gelsolin binds to actin filaments, promotes their severing in a calcium dependent manner, and efficiently caps filament barbed ends with nanomolar affinity. Up to eight calcium-binding sites with different affinities have been proposed to contribute to the calcium regulation. Binding of calcium opens the compact domain arrangement of inactive calcium-free gelsolin and reveals the actin binding regions in domains 1, 2 and 4. The calcium ions are grouped into type-1 calcium ions, which bind gelsolin and promote the opening of gelsolin domains, and type-2 calcium ions, which participate in actin binding (Choe et al., 2002). At sub-micromolar concentrations of calcium present in the cytoplasm, gelsolin exists in a partially activated form, which binds to actin filaments and slowly severs and caps the filament ends. At higher calcium concentrations (in the micromolar range), found in plasma, gelsolin becomes fully active and maximum severing is achieved. During calcium activation 1) gelsolin's compact domain arrangement opens, 2) Interactions between the N-terminus of G1, and C-terminus of G3, as well as the N-terminus of G4 and C-terminus of G6 become released, and 3) the calcium molecules occupy sites at the gelsolin/F-actin interface, thus facilitating the interaction. Binding of $PI(4,5)P_2$ inhibits barbed end capping by gelsolin and probably inactivates it near the plasma membrane (Choe et al., 2002; Burtnick et al., 2004).

The six gelsolin segments appear to have evolved from a single ancestral domain possibly by a gene triplication process, followed by a gene duplication event. All six segments share remote structural homology to the ADF-H domain family, with domains 1 and 4 being the most similar in terms of structure (Choe et al., 2002). During apoptosis, gelsolin is cleaved by caspases 3, 7, and 9 to produce a gelsolin fragment comprised only of domains 1-3. This fragment is independent of calcium regulation and proceeds to dismantle the actin cytoskeleton in an uncontrolled manner (Kothakota et al., 1997). However, the exact role of gelsolin in apoptosis remains a mystery. Further, a hereditary mutation in *gelsolin* (causing the change of Asp187 to Asn or Tyr) results in gelsolin fragmenting by furin and in subsequent assembly of the fragments into amyloid fibers. The fiber accumulation results in a range of neuropathies, ophthalmic disorders, and dermatological abnormalities, collectively termed Finnish-type familial amyloidosis (Burtnick et al., 2004; Levy et al., 1990; Maury et al., 1990).

1.4.3.2. Heterodimeric capping protein

Heterodimeric capping protein (CP) is another highly conserved actin filament barbed end binding protein. CP is found in nearly all eukaryotic cells, including fungi, plants, and virtually all cells and tissues of vertebrates. CP is also known as CapZ in skeletal muscle and Cap32/34 in *Dictyostelium*. It binds to actin filament barbed ends with nanomolar affinity and efficiently caps the filament ends by preventing actin monomer assembly or disassembly at the barbed end (reviewed in Wear and Cooper, 2004).

The α- and β-subunits of CP have molecular weights between 28-36 kDa. The structure of CP revealed a pseudo two-fold rotation symmetry between the two subunits, which share nearly identical secondary and tertiary structures despite almost complete lack of sequence identity. No other protein structures in the database resemble CP. The two-fold rotational

symmetry, together with biochemical evidence, gave rise to the 'tentacle' model of capping protein binding to the filament barbed end. In this model the interaction with the terminal actin subunits of the filament barbed end is mediated by two flexible 'tentacle' regions at the C-terminal ends of CP α- and β-subunits (Yamashita et al., 2003).

In bakers yeast, filament capping by capping protein is required for its correct function and localization *in vivo* (Kim et al., 2004). Most likely actin filaments (at least in yeast actin patches) are nucleated by the Arp2/3 complex, and grow for a limited time, after which they are capped. CP is a central component of the dendritic nucleation model, where Arp2/3 complex, WASP, ADF/cofilin, profilin, and capping proteins maintain the actin machinery in a metastable state, ready for directed polymerization (reviewed in Pollard et al., 2000). CP is also an essential component of the *in vitro* actin-based motility medium (Loisel et al., 1999). In this system, the free energy released by ATP hydrolysis linked to actin polymerization drives the actin based propulsion of functionalized microbeads. The proteins that are required for the bead motility are actin, Arp2/3 complex, N-WASP, a capping protein, ADF/cofilin, and profilin. The same proteins have also been shown to be essential for lamellipodium motility in mammalian cells (Rogers et al., 2003). Capping protein has also been reported to bind several other cellular components. $PI(4,5)P_2$, CARMIL, V-1, the membrane associated CD2AP protein, and CK2 protein kinase interation partner 1 (CKIP-1) directly bind CP and inhibit its capping activity (Uruno et al., 2006; Taoka et al., 2003; Bruck et al., 2006; Canton et al., 2006). Capping protein also binds to twinfilin and Enabled/ vasodilator-stimulated phosphoprotein (Ena/VASP) protein. However the latter proteins do not inhibit capping protein's capping activity and the exact biological function of these interactions remains unclear (reviewed in Wear and Cooper, 2004).

1.4.3.3. Other capping proteins

Other, less thoroughly characterized actin filament capping proteins include Eps8, actin interacting protein 1 (Aip1), and tropomodulins. Eps8 is a ~820 residue protein, consisting of a putative phosphotyrosine binding (PTB) domain, an SH3 domain, and a C-terminal effector region (Croce et al., 2004; Disanza et al., 2004). Eps8 family proteins are apparently found only from higher eukaryotes. In mammals, four isoforms with similar topologies are present. Eps8 forms a complex with Abi1 and Sos1, which promotes the activation of the small GTPase Rac. Eps8 proteins also bind to the sides of actin filaments through the C-terminal effector domain with an affinity of 0.45 μM. The effector domain is homologous to the sterile α-motif (SAM)/Pointed domain (reviewed in Higgs, 2004). Eps8 also caps actin filament barbed ends with nanomolar affinity (Disanza et al., 2004). However, neither the full-length Eps8 nor the effector domain possess the capping activity. Instead, this activity seems to localize to the central region of the molecule with the effector domain playing an assisting role in the capping process. Furthermore, it has been shown that Eps8 constructs, which cap actin filaments act as monomers, whereas the full-length protein exists as a dimer. This might mean that the full-length Eps8 normally exists in an autoinhibited state. Also, addition of Abi1 elicits the capping activity of Eps8, and thus Abi1 might function as an activator of Eps8. Thus Eps8 constitutes a new class of

actin filament barbed end capping proteins, characterized by the conserved C-terminal effector domain (Disanza et al., 2004).

Aip1 is a ~600 amino acid protein found in organisms from yeast to mammals. By itself Aip1 binds actin filaments very weakly, but addition of ADF/cofilin increases its affinity to actin filaments. In biochemical assays Aip1 increases the actin filament depolymerization activity of ADF/cofilin (reviewed in Ono, 2003). Recent studies proposed that Aip1 both severs actin filaments and caps their barbed ends with an ADF/cofilin dependent mechanism (Okada et al., 2002; Balcer et al., 2003). However, at the moment the biochemical basis and the possible *in vivo* relevance of actin filament barbed end capping by Aip1 is not clear.

Tropomodulins (Tmods) are ~40 kDa proteins found from several metazoan species (reviewed in Fischer and Fowler, 2003). Four isoforms of Tmod exist in most cells of vertebrates. Tmods specifically bind the pointed ends of actin filaments, and cap them with an affinity of 100-300 nM. Additionally, Tmods bind to tropomyosin molecules with an affinity of 0.2-1.0 μM. This binding strongly upregulates the pointed end capping activity of Tmod, which then caps the tropomyosin-coated actin filament pointed ends with an affinity of ≤0.05 nM (Fowler et al., 2003; Weber et al., 1999; Weber et al., 1994). Tropomyosins are elongated proteins, which can stabilize actin filaments by binding along the filament length (reviewed in Gunning et al., 2005). Thus, tropomodulins and tropomyosins could function together to protect filaments from depolymerization in both dynamic (for example actin-rich regions of motile cells) and very stable (the sarcomeres of muscle cells) actin filament structures in cells.

Also Ena/VASP proteins have been shown to inhibit the effect of capping proteins, and this might imply association at the barbed end and possible capping (Bear et al., 2002). However, direct capping activity by Ena/VASP proteins has not been demonstrated.

1.4.4. Actin monomer binding proteins

The cellular concentration of actin monomers (~100-300 μM in the cells of multicellular eukaryotes) (Pollard et al., 2000) greatly exceeds the concentration required for spontaneous actin polymerization (~0.1 μM). A variety of actin monomer binding proteins regulate the size, localization, and the dynamics of the large unpolymerized actin monomer pool (reviewed in Paavilainen et al., 2004). Six of these protein families are present throughout eukaryotic evolution and five of them will be presented in this chapter. The sixth member, twinfilin, will be presented in section 1.4.5.

1.4.4.1. Profilin

Profilin is a small (12-16 kDa) single-domain protein found from all eukaryotes (Figure 4). One profilin isoform is present in yeasts, whereas four tissue-specific isoforms have been identified in mammals (reviewed in Witke, 2004). Profilin forms a 1:1 stoichiometric complex with actin monomers and binds ATP-actin monomers with higher affinity (K_D = 0.1 μM) than ADP-actin monomers (K_D = 0.5 μM). The profilin-actin complex (Figure 4) is capable of associating to the actin filament barbed end. However, profilin inhibits the nucleation of actin filaments. Profilin accelerates the ADP-ATP exchange on actin monomer by ~1000-fold and this is probably the most important function of

this protein. Profilin also lowers the critical concentration for actin polymerization at the barbed end (reviewed in Yarmola and Bubb, 2006).

Profilin binds several proteins via their proline-rich sequences (reviewed in Witke, 2004). These proteins include VASP, N-WASP and the actin-filament-nucleating formin proteins. These interactions can potentially localize profilin, in complex with the actin monomer, to the sites of rapid actin assembly in cells. Other reported binding partners include Arp2/3, Mena, DFNA1, spinal muscular atrophy protein (SMN), drebrin, and gephyrin. Additionally, profilin is able to form a ternary complex with thymosin-β4 and an actin monomer. This interaction is predicted to further contribute to an increase in the amount of unpolymerized actin and to sequester free profilin in the cytoplasm. Profilin also interacts with phosphoinositides (mainly $PI(4,5)P_2$ and $PI(3,4,5)P_3$) and this interaction inhibits the actin monomer binding of profilin (reviewed in Witke, 2004).

1.4.4.2. Srv2/CAP

Suppressor of Ras/cyclase associated proteins (Srv2/CAP) are 50-60 kDa multifunctional proteins, apparently

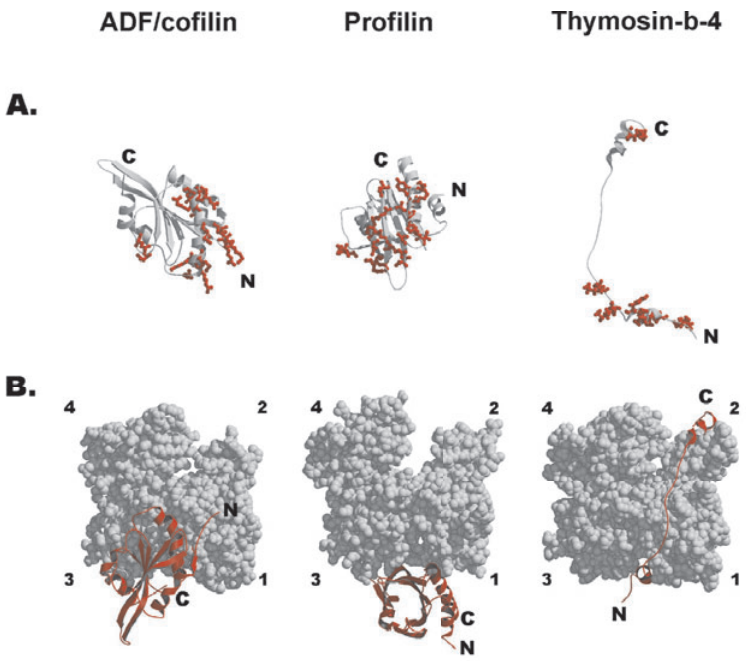


Fig 4. The structures and actin interactions of ADF/cofilin, profilin and thymosin-β4. A. Ribbon diagrams of the three proteins (PDB codes 1cof, 1hlu and 1uy5, respectively). The protein is displayed as a ribbons structure, and the residues known to be involved in actin interactions as red ball-and-sticks. **B.** Structures in complex with an actin monomer. Profilin and thymosin-β4 -structures have been experimentally solved (Schutt et al., 1993, Hertzog, 2004), whereas the ADF/cofilin-actin monomer -structure is a model derived from a molecular dynamics simulation (Wriggers et al., 1998). Actin is represented as a space-filling model, whereas the interacting proteins are displayed as ribbons. The picture was created with the programs Molscript (Kraulis, 1991) and RASTER3D (Merrit et al., 1994).

present in all eukaryotes (reviewed in Hubberstey and Mottillo, 2002). Based on genetic and biochemical evidence, Srv2/CAP plays an important role in several actin dependent processes such as cell polarity, cytokinesis, and endocytosis. Srv2/CAP proteins consist of an N-terminal alpha-helical domain that is responsible for binding adenylyl cyclase in yeast, followed by two proline-rich sequences that bind to the Src homology 3 (SH3) domain of Abp1 to localize yeast Srv2/CAP. These are followed by a WH2 domain, and a C-terminal actin monomer binding domain (reviewed in Hubberstey and Mottillo, 2002). The N-terminal adenylyl cyclase binding alpha-helical domain has been reported to form dimers (Mavoungou et al., 2004), and may contribute to the multimerisation of the full-length protein. The actin monomer binding domain strongly favors binding to ADP-actin monomers (K_D ~ 20 nM) over ATP-actin monomers (K_D ~ 1.5 μM) (Mattila et al., 2004). Structures of the C-terminal actin monomer binding domain from yeast and human reveal a novel type of parallel right-handed β-helix, which forms an intertwined dimer via exchange of a C-terminal β-hairpin. Competition between this domain and gelsolin segment-1 points to this domain binding in the proximity of actin subunits 1 and 3 (Dodatko et al., 2004) (Figure 2).

The purified full-length yeast protein exists as a stable multimer consisting of approximately six Srv2/CAP molecules, which have the capacity to bind to six actin monomers (Balcer et al., 2003). However, the molecular organization of the Srv2/CAP multimer is not presently well understood. It has also been shown that, together with ADF/cofilin, Srv2/CAP enhances actin filament turnover by enhancing the association rate of actin monomers at the filament barbed ends, and by promoting the nucleotide exchange rate on actin monomers (Moriyama and Yahara, 2002). Depletion of Srv2/CAP from *Dictyostelium*, *Drosophila*, and mammalian cells results in decreased actin filament turnover and formation of abnormal ADF/cofilin aggregates, (Bertling et al., 2004; Baum et al., 2000; Benlali et al., 2000; Noegel et al., 2004) suggesting that the *in vivo* function of Srv2/CAP would be to enhance actin filament turnover by recycling ADF/cofilin and actin molecules to regions of rapid actin filament disassembly and assembly, respectively.

1.4.4.3. WASP and WAVE

WASP (Wiscott-Aldrich syndrome protein) and WAVE (WASP family Verprolin homologous) proteins function presumably in all eukaryotic cells as activators of the Arp2/3 complex. Both proteins share a C-terminal catalytic VCA-domain (Verprolin homology, Central- and Acidic region) that mediates the direct interaction of these proteins with actin monomers and the Arp2/3 complex, and promotes Arp2/3 activation. The VCA domain consists of a WASP homology domain 2 (WH2), a central domain, and an acidic region. The WH2 domain and the central domain bind to actin monomers and to the Arp2/3 complex, whereas the acidic region binds only to Arp2/3 and upon binding causes a large conformational change of the complex, resulting in Arp2/3-induced actin filament nucleation (reviewed in Stradal et al., 2004; Stradal and Scita, 2006).

WASPs and WAVEs are ~500 amino acid proteins. In addition to the VCA region, common to all WASP and WAVE proteins, WASP and N-WASP have an N-terminal WASP homology 1 (WH1)

domain and a central GTPase binding domain (GBD). WASP and N-WASP exist in an autoinhibited state, which can be released by direct binding of Cdc42 and other factors, including PI(4,5)P_2, WASP interacting protein (WIP), and SH3 domain containing adaptor proteins such as Nck and Gbr2. Until recently, it was thought that N-WASP exists as an auto-inhibited monomer that would be activated by the binding of effector proteins (Prehoda et al., 2000). However, more recently it was found that the majority of N-WASP in cells is bound to WIP (WASP interacting protein) family proteins and that this interaction causes stabilization of the inactive conformation of N-WASP (Ho et al., 2001; Ho et al., 2004; Anton et al., 2002). This suggests that the WIP - N-WASP complex would represent the most relevant auto-inhibited state of N-WASP. An additional level of regulation was recently revealed by the finding that the binding of Toca-1 (Transducer of Cdc42-dependent actin assembly) to the WIP - N-WASP complex was required to complete the Cdc42-induced activation of N-WASP (Ho et al., 2004). To make matters even more complicated, Toca-1 belongs to a highly conserved three-gene family of proteins, all of which are probably able to bind the WIP/N-WASP complex, and also Toca-1 alone is able to directly bind Cdc42 (Ho et al., 2004). In addition, WASP and N-WASP can be phosphorylated on two residues in the VCA-region to modulate its affinity for the Arp2/3 complex (Cory et al., 2003). Thus, it seems that the regulation of N-WASP is much more complicated than previously thought and many more details are likely to remain to be revealed.

In contrast to the WASP family proteins, WAVE proteins (also called SCAR proteins) contain a unique WAVE homology domain (WHD) in place of the WH1 and GBD domains (reviewed in Stradal et al., 2004; Stradal and Scita, 2006). Three WAVE isoforms exist in mammals (WAVE-1, -2 and -3). Unlike the WASP proteins, WAVE proteins are not autoinhibited, but instead exist in a constitutively activated state which is repressed by the binding of effector proteins. Multiple proteins are known to bind to WAVE proteins and controversy exists about the effects of these proteins in regard to WAVE activation (reviewed in Stradal and Scita, 2006). However, it seems that in cells WAVE exists in stable complexes with effector proteins, which include at least HSPC300, Abi1 and Nap1 (Gautreau et al., 2004). All these proteins have been observed to rapidly relocalize to the leading edge of cells together with WAVE in response to Rac activation (Steffen et al., 2004), suggesting that in cells these proteins move as a stable complex. WAVE has also been shown to bind PI(3,4,5)P_3 which could also regulate its activity (Oikawa et al., 2004). Regulation of WAVE proteins appears to be as complex as that of WASP proteins. It is far from well understood, and seems to involve crosstalk with regulation of the WASP proteins.

1.4.4.4. Verprolin/WIP

Verprolin/WIP (WASP interacting protein) proteins are multifunctional proteins found in all eukaryotes with the exception of plants. One isoform (called verprolin for very proline-rich protein) is found in yeast, and three members in vertebrates that are called WIP, CR16 (Glucocorticoid-regulated gene product) and WIRE (WIP-related, also known as WICH). These proteins range in sizes between 450 to 800 amino acids. These three proteins differ in their tissue distribution. WIP is widely

expressed, most highly in hematopoietic cells, whereas CR16 and WIRE are expressed more tissue-specifically (reviewed in Anton and Jones, 2006; Aspenstrom, 2005).

Despite the difference in size, all WIP family proteins share a highly similar domain organization. The N-terminus of WIP proteins contains several proline-rich motives, which bind to profilin. Some of the proline-rich regions also appear to bind other proteins via their SH3 domains. These include cortactin, hematopoietic cell kinase (Hck), and the adaptor protein Nck. Following the proline-rich region, two WH2 domains facilitate the G- and F-actin binding ability of WIP proteins. The WASP binding motif in the C-terminus is responsible for interacting with WASP family proteins via their WH1 domains. Patients with Wiscott Aldrich Syndrome (WAS) have mutations in this part of WASP, which abolishes the WASP/WIP -interaction. This demonstrates the *in vivo* importance of the interaction. Additionally, a proline-rich region immediately preceding the WASP binding motif has been shown to participate in the WIP - WASP interaction (reviewed in Anton and Jones, 2006; Aspenstrom, 2005).

WIP, but not CR16, inhibits N-WASP -induced Arp2/3 mediated actin polymerization *in vitro*. WIP is also able to inhibit Cdc42-induced N-WASP activation in the absence of $PI(4,5)P_2$. In cells, WIP and WASP appear to function together at least in promoting filopodia formation (Ho et al., 2001; Martinez-Quiles et al., 2001).

WIP and WIRE proteins also inhibit actin depolymerization. This has provoked models where WIP proteins serve to protect certain filaments from depolymerization or severing in cells. WIP also interacts with cortactin, and actuates the Arp2/3 activating ability of cortactin. Based on this, it has been proposed that WIP could function in recruiting actin monomers to the cortactin-Arp2/3 -complex for actin filament nucleation/polymerization (Kinley et al., 2003). *Vaccinia* virus has also been shown to utilize WIP to assemble actin comet tails for viral movement (Frischknecht et al., 1999; Moreau et al., 2000). WIRE and CR16 also bind Tuba, a protein linking the GTPase dynamin to the actin cytoskeleton (Salazar et al., 2003). Thus the cellular role of WIP is probably connected to regulation of actin polymerization events during endocytosis and vesicle transport in cells.

1.4.4.5. ADF/Cofilin

ADF/cofilins are small (15-20 kDa) proteins, consisting of a single actin depolymerizing factor -homology (ADF-H) domain (Figure 4). ADF/cofilins are abundant proteins found in all eukaryotes. In most organisms, like yeast, *Drosophila*, and plants, these proteins are essential for viability (reviewed in Bamburg, 1999). Mutations in yeast cofilin result in the accumulation of abnormal actin structures in cells, demonstrating the importance of this protein in actin dynamics (Lappalainen et al., 1997).

ADF/cofilins preferably bind to ADP-actin monomers (K_D = 0.09-0.19 μM for ADP-actin monomers and 6.0-7.0 μM for ATP-actin monomers) (Ressad et al., 1998). They also cooperatively bind the sides of ADP-actin filaments, and enhance the pointed end depolymerization of these filaments by a factor of ~25 (Carlier et al., 1997). The binding of ADF/cofilin to the filament side induces a twist in the actin filament (~5° per subunit) and this twisting probably alters the thermodynamic properties of the actin filament and leads to enhanced filament depolymerization at the

pointed end (Galkin et al., 2001; McGough et al., 1997). Increasing the pointed end depolymerization rate is believed to be the main function of ADF/cofilin in cells (Hotulainen et al., 2005).

In addition to the F-actin depolymerizing activity, ADF/cofilins also promote the dissociation of inorganic phosphate from actin filaments (Blanchoin and Pollard, 1999). Upon monomer assembly, the actin-bound ATP is rapidly hydrolyzed after which the γ-phosphate of ATP diffuses out of the filament. This diffusion is slower than the rate of filament elongation and ATP hydrolysis, and thus cells need additional factors to speed up phosphate diffusion. In addition to these activities, ADF/cofilins also possess a weak filament severing activity and inhibit spontaneous nucleotide exchange on actin (Hawkins et al., 1993; Andrianantoandro and Pollard, 2006).

ADF/cofilin activity is regulated by phosphorylation, pH, and binding of phosphoinositides and regulatory proteins (reviewed in Bamburg, 1999). Phosphorylation of a conserved N-terminal serine of ADF/cofilin, results in downregulation of its actin binding activity. ADF/cofilin can be phosphorylated by the kinases LIM and TESK, and specifically dephosphorylated by the Slingshot and Chronophin phosphatases (Niwa et al., 2002; Soosairajah et al., 2005; Gohla et al., 2005). Also binding to phosphoinositides downregulates the activity of ADF/cofilins, however the physiological importance of this interaction remains unknown (Yonezawa et al., 1990).

1.4.5 Twinfilin

1.4.5.1. Primary structure and evolution

Twinfilin is a highly conserved protein composed of two ADF-H domains (called Twf-N and Twf-C), separated by a ~30 residue linker region, and followed by a ~35 residue tail region (Figure 5A) (Lappalainen et al., 1998; Goode et al., 1998). The two ADF-H domains are ~20% identical to each other and to proteins of the ADF/cofilin -family. Twinfilin was originally identified from yeast (Goode et al., 1998) and has later been found from a wide variety of eukaryotes, with the exception of plants. The individual ADF-H domains are more highly conserved across species than individual N- or C-terminal domains within a species. This suggests that the two domains arose by a gene duplication of an ancient single-domain, ADF/cofilin-like protein before the divergence of the fungal and animal lineages.

1.4.5.2. Expression of twinfilin in cells and tissues

Only one twinfilin isoform has been found from yeast, whereas mammals have two isoforms, named twinfilin-1 and -2 (Vartiainen et al., 2003). The deletion of twinfilin in yeast results in the formation of somewhat abnormal actin patches and defects in the bipolar bud-site selection pattern (Goode et al., 1998). In mice, twinfilin-1 is expressed in most tissues (although not in skeletal muscle), with the strongest expression in liver and kidney. Twinfilin-2, on the other hand, is only very weakly expressed in non-muscle tissues, but is strongly expressed in heart and skeletal muscle. Either twinfilin-1 or -2 is present in most, if not all, cell types in mice, both during development and in adult mice (Vartiainen et al., 2003). In *Drosophila*, twinfilin is widely expressed during development and at least in ovarian cells it localizes to the cytoplasm and the

plasma membrane. Depletion of twinfilin from *Drosophila* results in a small body size and reduced activity of the mutant flies. In addition, the mutants have a rough eye phenotype and display severely disorganized sensory bristles (Wahlstrom et al., 2001). The bristles are long single cells, which are supported by a highly organized array of actin bundles. The development of these large cells is highly dependent on an intact actin cytoskeleton, and thus changes in their morphology often reflect major defects in actin regulation (Tilney and DeRosier, 2005).

1.4.5.3. Cellular roles of twinfilin

In yeast, twinfilin is a component of the actin patches (Figure 5B) (Goode et al., 1998). These patches are dynamic assemblies of

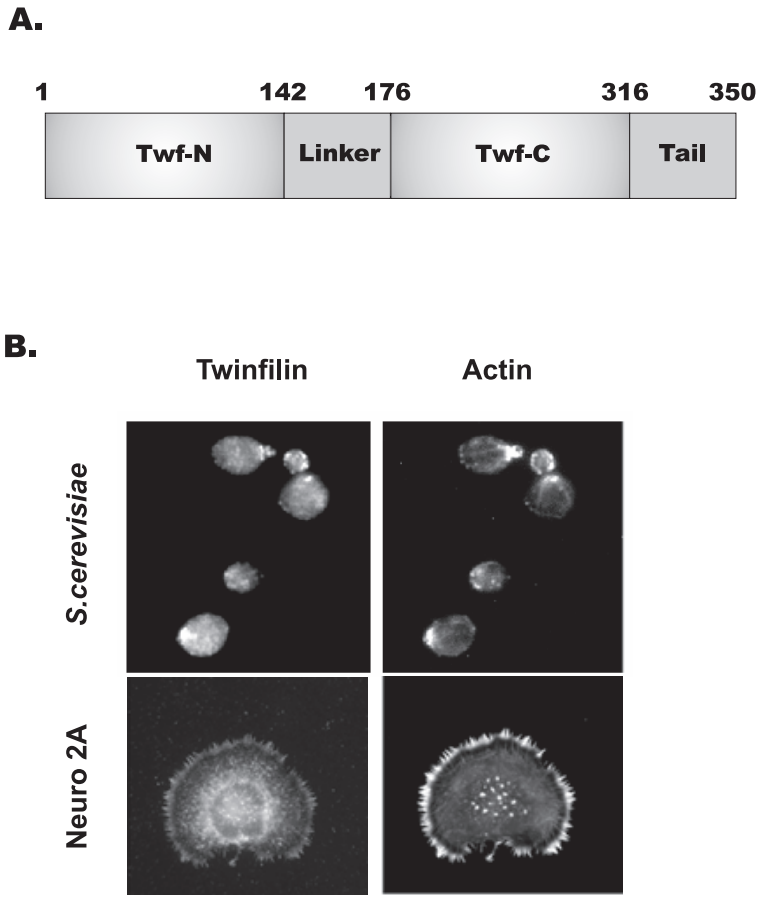


Fig 5. Domain structure and cellular localization of twinfilin. A. Twinfilin is composed of two ADF-H domains, called Twf-N and Twf-C. The two domains are separated by a conserved ~30 residue-long linker region, and Twf-C is followed by a C-terminal ~35 residue tail region. **B.** Twinfilin localizes to the dynamic actin patches in yeast cells modified from (Palmgren et al., 2001), and mouse twinfilin displays a punctate cytoplasmic localization pattern, but also localizes to the actin-rich lamellipodium in cultured non-muscle mammalian cells (modified from Vartiainen et al., 2003).

actin, and several actin regulators, at the sites of clathrin-coated pits immediately prior to vesicle release (Kaksonen et al., 2005). Several proteins implicated in endocytosis or actin regulation show dynamic spatiotemporal localization to actin patches, and actin polymerization is required for internalization of the clathrin coat during clathrin-mediated endocytosis (Newpher et al., 2005; Kaksonen et al., 2005). It has also been suggested that actin polymerization and/or myosin action could facilitate the scission of the endocytotic vesicle, instead of dynamin (Liu et al., 2006). The ability of twinfilin to bind actin monomers is required for its correct localization to actin patches (Palmgren et al., 2001). In mammalian cells, twinfilin shows a strong, punctate, perinuclear localization and also localizes to actin-rich processes (Figure 5B) (Vartiainen et al., 2003). More recently twinfilin was also shown to localize to the actin tails of endocytic vesicles, further suggesting a role for twinfilin in endocytosis (Helfer et al., 2006). It has also been shown that depletion of twinfilin results in defects in the sub-cellular localization of endocytic vesicles (Pelkmans et al., 2005). Also overexpression of individual ADF-H domains of twinfilin in cells caused a decrease in endocytic uptake, indicating that these domains were interfering with the function of the endogenous full-length protein in regulating actin dynamics at the actin tails of the vesicles (Helfer et al., 2006).

1.4.5.4. Biochemical properties of twinfilin

Twinfilin binds actin monomers with high affinity (K_D for ADP-G-actin ~70 nM), inhibits nucleotide exchange on the actin monomer, and prevents actin assembly to filament ends. Although twinfilin is composed of two ADF-H domains, it appears to form a 1:1 complex with actin monomers (Goode et al., 1998; Vartiainen et al., 2000; Palmgren et al., 2001). Given the relatively high concentration of twinfilin in cells (~1:10 to actin in yeast cells) it is probable that a large number of the actin monomers in cells are complexed with twinfilin, preventing them polymerizing. Thus twinfilin appears to be involved in the regulation of the large unpolymerized actin pool in cells (Palmgren et al., 2001).

Twinfilin also associates with the heterodimeric capping protein (CP) and at least in yeast cells this interaction is required for the correct sub-cellular localization of twinfilin. However the biological significance of this interaction is not clear. Twinfilin also binds to certain phospholipids, mainly to $PI(4,5)P_2$, and this interaction inhibits its ability to bind to and sequester actin monomers (Palmgren et al., 2001).

Recently, twinfilin was shown to cap actin filament barbed ends with high affinity (0.2 μM and 0.013 μM for ATP- and ADP-actin filaments, respectively). Twinfilin could also replace CP or gelsolin in a biomimetic bead motility assay, confirming that it caps actin filament barbed ends during motile processes. Neither of the isolated ADF-H domains was able to cap actin filament barbed ends (Helfer et al., 2006). It was also recently demonstrated that yeast twinfilin was able to sever actin filaments at low pH. However, no severing was detected at physiological pH and therefore the physiological relevance of this activity remains unclear (Moseley et al., 2006).

2. AIMS OF THE STUDY

Twinfilin is a recently identified, evolutionarily conserved, actin monomer binding protein. Because twinfilin's role in the regulation of actin dynamics and the mechanism of its interaction with actin and capping protein have been poorly understood, we examined the biochemical, cell biological, and structural properties of twinfilin. The specific aims of this study were to:

1. Elucidate the roles of the two ADF-H domains of twinfilin during interaction with the actin monomer and filament barbed ends.

2. Determine the structure of twinfilin or its isolated ADF-H domains and map their actin binding sites.

3. Understand the molecular mechanism of the twinfilin - capping protein interaction.

4. Reveal the mechanism of the actin filament barbed end capping activity of twinfilin.

3. METHODS

The methods that I used in this study are summarized in the table below. Detailed descriptions of the methods are found in the publications.

Method	Publication
Recombinant DNA techniques	I, II, III, IV
SDS-PAGE (Laemmli, 1970)	I, II, III, IV
Recombinant protein production and purification (Vartiainen et al., 2003)	I, II, III, IV
Purification of rabbit muscle actin (Spudich and Watt, 1971)	I, II, III, IV
NBD-G-actin binding assay (Carlier et al., 1997)	I, II, IV
Pyrenyl-actin assembly/disassembly assay (Helfer et al., 2006)	IV
Biomimetic bead motility assay (Helfer et al., 2006; Wiesner et al., 2003)	IV
Protein crystallography	II
Small angle x-ray scattering	III
Actin filament co-sedimentation assay	I, IV
Urea denaturation assay	II
Native gel electrophoresis (Palmgren et al., 2001; Safer, 1989; Maciver and Weeds, 1994)	III

E.coli expression plasmids used in this study.

Plasmid	Expression construct	Reference
pPL78	Full-length GST-Mouse Twinfilin-1 (mTwf)	(Vartiainen et al., 2000)
pPL144	Full-length His-Mouse Twinfilin-1	I
pPL81	GST-mTwf$_{1-142}$	I
pPL82	GST-mTwf$_{1-174}$	I
pPL83	GST-mTwf$_{141-350}$	I
pPL84	GST-mTwf$_{169-350}$	I
pPL89	GST-mTwf$_{141-322}$	I
pPL419-424	GST-mTwf$_{1-174}$ point mutations	II
pPL145	Mouse capping protein$_{\alpha1\beta2}$	III
pPL224	His-mTwf$_{169-322}$	IV
pPL400-405	His-mTwf$_{169-322}$ point mutations	IV
pPL406	Non-tagged mTwf$_{176-316}$	IV
pPL407-413	His-Mtwf domain-swap mutations	IV

4. RESULTS AND DISCUSSION

4.1. Interaction of twinfilin with actin monomers (I+II)

4.1.1. The roles of twinfilin's ADF-H domains in actin monomer binding (I)

Before this study, it was known that twinfilin binds actin monomers, but was thought not to interact with actin filaments (Vartiainen et al., 2000; Goode et al., 1998). In order to reveal the roles of Twf-N and Twf-C in twinfilin's interaction with actin monomers, we performed a deletion analysis of twinfilin. We cloned, expressed in *E.coli*, and purified deletion constructs with twinfilin's ADF-H domains with or without the linker and tail regions. Wild-type and mutant proteins were then tested for G-actin binding. These assays revealed that the full-length mouse twinfilin-1 binds ADP-actin monomers with much higher affinity (K_D ~70 nM) than ATP-actin monomers (K_D ~0.5 μM) (I). It is important to note that also ADF/cofilin proteins preferably bind ADP-actin (Ressad et al., 1998), and twinfilin competes with ADF/cofilin for actin monomer binding (I). This suggests that these two proteins bind to the same site on the actin monomer. It should also be noted that twinfilin binds ADP-G-actin with a significantly higher affinity than other known actin sequestering proteins, profilin (K_D 0,5-3 μM)(Vinson et al., 1998) and thymosin-β4 (K_D 80-100 μM) (Carlier et al., 1993). Therefore, it seems that twinfilin may sequester a significant proportion of ADP-actin monomers in cells.

Despite its two actin-binding domains, twinfilin forms a 1:1 stoichiometric complex with actin monomers (Goode et al., 1998; Vartiainen et al., 2000). Our studies showed that both Twf-N and Twf-C bind actin monomers (K_D ~0.70 μM and ~0.07 μM for Twf-N and Twf-C, respectively), with the C-terminal domain being the high-affinity G-actin binding domain. The conserved linker and tail regions had no effect on the G-actin binding activity of twinfilin, indicating that they play a role in twinfilin functions other than ADP-G-actin sequestration (I).

The association of twinfilin with ADP-G-actin follows a biphasic binding scheme, suggesting that upon binding to G-actin, Twf-N initially associates with the actin monomer, after which the monomer is transferred to the high-affinity C-terminal domain (I). Thus Twf-N appears to act as the 'entry-site' during the initial G-actin binding event, and Twf-C provides the main binding affinity required for formation of the stable twinfilin - actin monomer - complex. Taken together, the data obtained suggest that twinfilin could function in maintaining the large unpolymerized actin monomer pool in cells by competing ADP-G-actin monomers away from ADF/cofilin, preventing their nucleotide exchange, and subsequent assembly into filaments.

4.1.2 Structure and G-actin binding interface of Twf-N (II)

In order to understand why twinfilin binds only G- and not F-actin, and to elucidate how the individual domains contribute to the G-actin binding activity of the full-length protein, we performed crystallization trials for Twf-N and Twf-C. We obtained crystals from Twf-N that were suitable for x-ray data collection. The crystals belonged to the crystallographic spacegroup $P2_12_12_1$ and diffracted to 1.6 Å resolution at the beamline BW7B of the European Molecular Biology Laboratory in

Hamburg, Germany. The structure of Twf-N was solved using the single anomalous dispersion (SAD) method with data measured from a gold-derivative crystal of Twf-N. The structure was homologous to the already solved structures of ADF/cofilins (Figure 6 and II) (Hatanaka et al., 1996; Fedorov et al., 1997; Leonard et al., 1997; Bowman et al., 2000) with two clear differences: 1) The long beta-extension of ADF/cofilin, implicated in F-actin binding of ADF/cofilin, was clearly bent in Twf-N, and 2) the most C-terminal α-helix (also involved in interactions with F-actin in ADF/cofilin) was slightly bent in Twf-N (II). The mutagenesis study of Twf-N revealed that it utilizes a similar, but more extended, binding site for G-actin than ADF/cofilin (II). The largest differences were observed in regions that in ADF/cofilin are known to be important for F-actin binding (Lappalainen et al., 1997), providing a structural explanation to why Twf-N can not bind to actin filaments. More recently, structures of F-actin binding domains from coactosin and Abp1 have been determined (Quintero-Monzon et al., 2005; Hellman et al., 2004). Also these ADF-H domains display close structural similarity to ADF/cofilins and Twf-N, although they differ from Twf-N in the above-mentioned regions. This provides further support for the critical role of these ADF-H domain regions in F-actin binding.

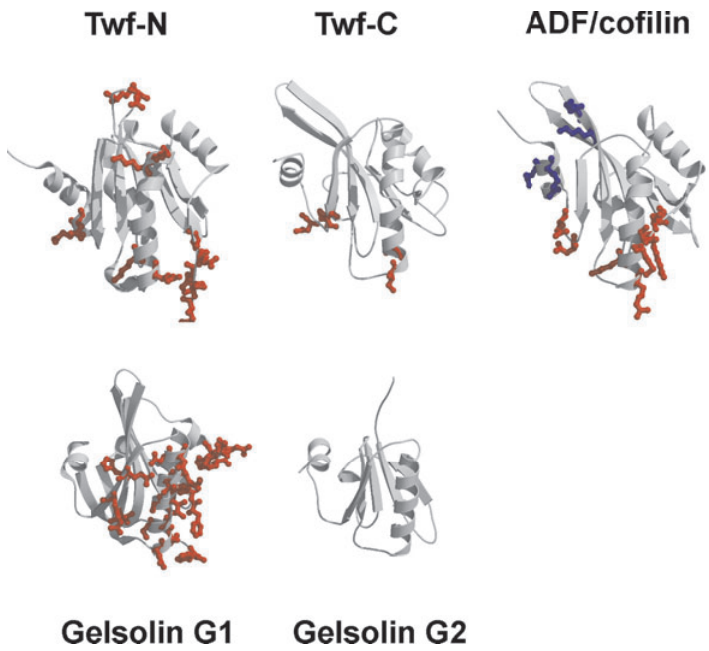


Fig 6. The structures and known actin binding sites of Twf-N, Twf-C, ADF/cofilin, and gelsolin domains 1 and 2 (PDB codes 1m4j, 2hd7, 1cof, and 1rgi (G1 and G2), respectively). The residues known to be important actin monomer interactions (Lappalainen et al., 1997; McLaughlin et al., 1993) (and this study) are displayed as red ball-and-sticks. Residues important for the interaction of ADF/cofilin with actin filaments (Lappalainen et al., 1997) are displayed in blue. The picture was created with the programs Molscript (Kraulis, 1991) and RASTER3D (Merrit et al., 1994).

4.2. The mechanism of twinfilin - capping protein interaction (III)

4.2.1. Biochemical characterization of twinfilin - capping protein interaction (III)

Twinfilin interacts with the heterodimeric capping protein (CP) and this interaction is required for twinfilin's correct sub-cellular localization in yeast cells (Palmgren et al., 2001). In our study we examined the biological role and the molecular mechanism of this interaction. By systematic mutagenesis, we discovered that the CP-binding site of twinflilin resides in its C-terminal tail region (III). Pyrene-actin assembly assays revealed that the association of twinfilin with CP did not affect CP's actin filament barbed end capping activity (III). Furthermore, the binding of CP to wild-type twinfilin in solution did not affect the actin monomer binding activity of twinfilin (III). Thus, twinfilin is not a regulator of the capping activity of CP, unlike the other so far characterized CP-binding proteins CARMIL, V-1, CD2AP, and CKIP-1 (Uruno et al., 2006; Taoka et al., 2003; Bruck et al., 2006; Canton et al., 2006). However, our studies were performed with soluble CP, and it is possible that filament-bound CP might affect the binding of twinfilin to G-actin, because the high-affinity C-terminal domain of twinfilin resides close to the CP binding C-terminal tail region of twinfilin.

4.2.2. Structure of the twinfilin - capping protein complex (III)

A solution structure of the twinfilin - CP -complex was obtained by using small-angle x-ray scattering (SAXS) (III). SAXS is a powerful tool for obtaining information about the gross structural features of biological macromolecules such as shape, quaternary and tertiary structure (Svergun et al., 2001). This method provides only low-resolution information about the studied macromolecules, but is fast and requires only dilute samples for the measurement. SAXS data were collected with solution samples of isolated mouse twinfilin-1, CP, and the twinfilin - CP -complex at beamline 2.1 of the Synchrotron Radiation Source (SRS), Daresbury, UK. Subsequently, low-resolution models of the proteins were generated *ab initio*. The obtained structures revealed that the full-length twinfilin is an elongated two-domain structure with a kinked linker region separating the two domains (III). The model for CP revealed an asymmetric particle, approximately 9 nm in length (III). This corresponds well with the recently determined crystal structure of the heterodimeric capping protein (Yamashita et al., 2003). The model for the twinfilin - CP -complex showed an asymmetric particle connected to a two-domain structure. Fitting of atomic coordinates of Twf-N (in the place of both domains, as the structure of Twf-C was not yet available), and CP in the low-resolution 'dummy' atom models provided a working model of the twinfilin/CP complex (III). Later the structure of Twf-C was positioned in this model (Figure 7C). The model confirmed that twinfilin utilizes the C-terminal tail region for interacting with CP, and that the N-terminal domain does not contribute to the interaction (III). The structure supports the idea that twinfilin binding to filament-bound CP would affect the G-actin binding activity of twinfilin, given the proximity of the high-affinity CP- and G-actin -binding sites in the obtained twinfilin - CP solution structure (Figure 7C).

4.2.3. Cell biological role of the twinfilin - capping protein interaction (III)

Expression of yeast twinfilin mutants, unable to bind CP, confirmed that this activity is required for the correct sub-cellular localization of twinfilin to yeast actin patches. Furthermore, yeast strains expressing the mutant proteins with defects in actin monomer or CP binding showed synthetic lethality when crossed with certain cofilin and profilin mutations (III), similar to Δ*twf1* cells (Palmgren et al., 2001). This suggests that both activities are required for the correct *in vivo* function of twinfilin in actin regulation. However CP does not require twinfilin for its localization, since CP localizes normally in twinfilin-deletion cells (III).

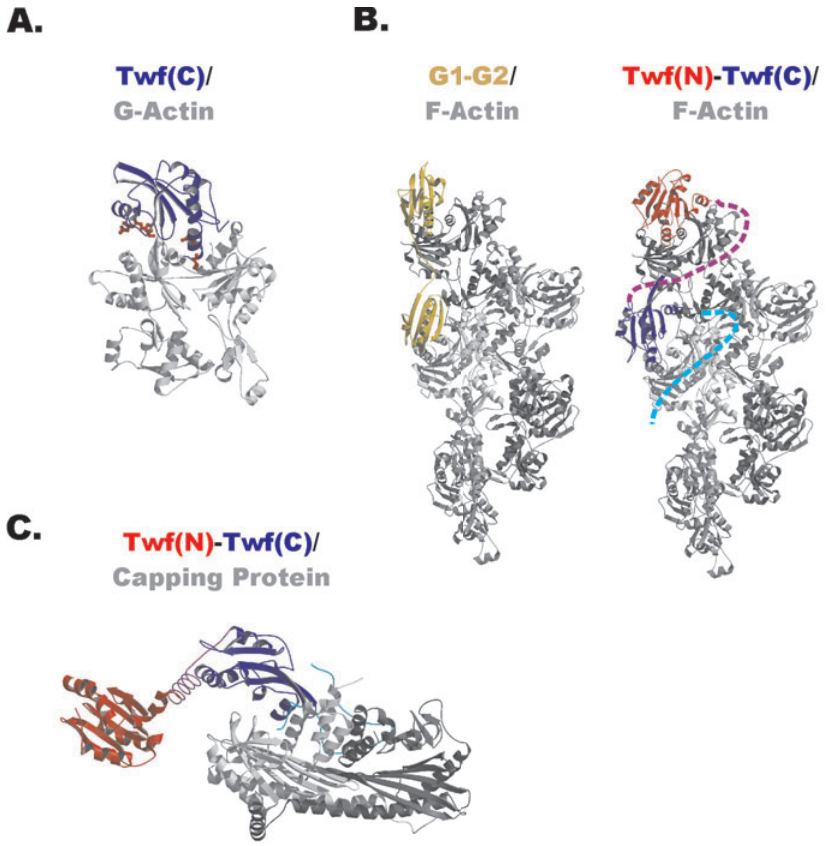


Fig 7. Structural models of twinfilin with its binding partners. A. A model for interaction of the high-affinity Twf-C with the actin monomer. Twf-C was positioned in the place of ADF/cofilin in the ADF/cofilin-actin monomer -model (Wriggers et al., 1998). **B.** The 'Holmes'-model of the actin filament (Holmes et al., 1990) with gelsolin G1-G2 fitted onto the barbed end (Burtnick et al., 2004) (left). A model of a twinfilin-capped actin filament barbed end from this study (right). **C.** A model of the twinfilin-capping protein -complex. Atomic structures of Twf-N, Twf-C and capping protein were manually positioned on the 'dummy atom' -models obtained *ab initio* from small-angle scattering studies (IV). The picture was created with the programs Molscript (Kraulis, 1991) and RASTER3D (Merrit et al., 1994).

Twinfilin shows diffuse cytoplasmic localization, but is also concentrated to actin-rich structures in yeast, *Drosophila* and mouse cells (Palmgren et al., 2001; Wahlstrom et al., 2001; Vartiainen et al., 2003). Therefore it is possible that the cellular role of twinfilin is to sequester ADP-actin monomers from the cytoplasm, and deliver them to regions of rapid actin assembly (e.g. yeast actin patches or lamellipoda of mammalian cells) via direct interactions with CP. Binding to the filament-bound CP could cause twinfilin to release the actin monomer, enabling nucleotide exchange on actin, and assembly to the free filament barbed ends. Because both twinfilin and CP are abundant proteins in yeast, and the majority of CP is localized to yeast actin patches (Palmgren et al., 2001; Kim et al., 2004), delivery of actin monomers to actin patches by twinfilin could have a significant contribution to increasing actin filament turnover in yeast cells. This hypothesis is also supported by the finding that a strong hypomorphic *twinfilin* mutation in *Drosophila* results in the accumulation of displaced actin filament structures in the flies bristle cells (Wahlstrom et al., 2001). Together these data indicate that twinfilin does not function only as an actin monomer sequestring protein, but requires the CP interaction for its biological activity (III).

4.3. Molecular mechanism of barbed end capping by twinfilin (IV)

During this thesis work it was reported that, in addition to actin monomer sequestering and CP binding activities, twinfilin also efficiently caps actin filament barbed ends (Helfer et al., 2006). Therefore we investigated the molecular mechanism behind this interaction.

4.3.1. Structure of Twf-C and its G-actin binding site (IV)

After failing to produce diffraction-quality crystals of Twf-C, we decided to solve the structure of Twf-C by NMR spectroscopy. For this purpose, we produced a ^{13}C- and ^{15}N-labeled sample of mouse twinfilin-1 residues 176-316 (Twf-C). Triple resonance spectra were acquired from a 1 mM sample of Twf-C at 25 ^{o}C. The structure of Twf-C shows a typical ADF-H -fold with a long β-extension, formed by β-sheets 3 and 4 (IV). This closely resembles the β-extension of ADF/cofilin and is clearly different from the one of Twf-N (Figure 6 and IV). Therefore, we next tested whether Twf-C could also bind F-actin in addition to its G-actin binding activity (I). Actin filament co-sedimentation assays showed that Twf-C, but not Twf-N, binds to actin filaments (IV). Additionally, Twf-C increased the pointed-end depolymerization rate of actin filaments (IV), like ADF/cofilin (Ressad et al., 1998), indicating that Twf-C interacts with the sides of actin filaments in a manner similar to ADF/cofilin. Also the C-terminal tail of twinfilin, previously shown to interact with CP (III), dramatically increased the affinity of Twf-C for F-actin (IV), indicating that also the tail region is involved in the Twf-C/F-actin interaction.

4.3.2. Biochemical characterization of the roles of Twf-N and Twf-C in barbed end capping (IV)

Next we focused on the roles of Twf-N and Twf-C during twinfilin's newly characterized barbed end capping activity (Helfer et al., 2006). For this purpose, we generated a set of domain swap/inactivation mutants of full-length twinfilin. All mutants

were designed such that they retained the original linker and tail regions of twinfilin. The purified mutant proteins were assayed for barbed end capping with a pyrene actin filament growth assay and with a biomimetic bead motility assay (Loisel et al., 1999). The bead motility assay consists of five proteins, which are necessary for actin based motility in cells. These are N-WASP, Arp2/3, ADF, profilin, and capping protein. Addition of these proteins into a solution of F-actin with N-WASP coated silica microbeads causes the beads to move at velocities up to 8 μm/min. Without a capping protein (gelsolin, heterodimeric capping protein or twinfilin) the beads show no directed movement and fail to form the characteristic actin tails (Loisel et al., 1999). These assays showed that the presence of both functional twinfilin domains is required for barbed end capping, and that the binding affinity of Twf-C to F-actin is critical for capping (IV). The C-terminal tail region was shown to increase the capping affinity of full-length twinfilin, demonstrating the importance of the filament side-binding activity of Twf-C for the barbed end capping (IV). Twf-N did not display any detectable F-actin binding activity. However, a construct comprised of two Twf-N domains was able to very weakly cap filament barbed ends, indicating that Twf-N could associate with filament barbed ends in a different manner than ADF/cofilin (IV). Twf-N also shows high structural homology to gelsolin segment-1 (G1) (IV and Figure 6), which binds to the terminal actin monomer when gelsolin caps actin filaments (Burtnick et al., 2004). Therefore, it is likely that Twf-N binds to the terminal actin monomer at the actin filament barbed end.

4.3.3. A general mechanism of barbed end capping by twinfilin and gelsolin and the evolution of this activity (IV)

The six domains of gelsolin (G1-G6) are structurally homologous to the ADF-H domains of twinfilin (Figure 6), although ADF-H domains and gelsolin domains do not display detectable sequence homology to each other (Choe et al., 2002). It is also believed that the ADF-H and gelsolin domains bind actin through similar binding interfaces (Wriggers et al., 1998; Dominguez, 2004). This, and the fact that the minimal gelsolin fragment able to cap barbed ends consists of the first two domains (G1-G2), led us to consider whether twinfilin might cap filament barbed ends in a similar fashion to gelsolin. Like gelsolin domain 1 (G1), Twf-N binds only actin monomers (IV). Furthermore, gelsolin domain 2 (G2) binds both actin monomers and filaments, as Twf-C (IV). Because of these biochemical and structural similarities, we conclude that twinfilin and gelsolin most likely cap actin filament barbed ends via a similar mechanism.

Based on the model of a G1-G2 - capped actin filament barbed end (Burtnick et al., 2004), we superimposed the atomic coordinates of Twf-N and Twf-C in the place of G1 and G2 to obtain a model of the twinfilin-capped filament barbed end (Figure 7B and IV). It should be noted that the different orientation of the β-protrusion, formed by β-sheets 3 and 4 of Twf-N, causes a steric clash with F-actin if Twf-N is superimposed on the structure of G2 in the model. This provides a structural explanation why Twf-N only binds to actin monomers (and to the filament end). Twf-C can be placed either on the side of the filament or on the terminal monomer (IV).

Together with the biochemical data obtained, this model provides a structural explanation for the unique biochemical and structural properties of the two twinfilin domains during barbed end capping (IV). We propose that the barbed end capping activities of twinfilin and gelsolin arose from gene duplications of ancient ADF/cofilin- and gelsolin-like domains, respectively. In support of this scheme, we generated a hybrid protein, consisting of two cofilin-2 molecules, connected by the twinfilin linker region. Cofilin-2 protein is able to bind to actin monomers and filaments and is very distantly related to the twinfilin domains (~15% sequence identity to Twf-N or Twf-C). Surprisingly, this hybrid protein was able to cap actin filaments (IV). This finding supports our hypothesis that twinfilin and gelsolin cap actin filaments via a similar mechanism and that during evolution these proteins independently obtained this activity by parallel duplications of ancient G- and F-actin binding domains. Later, both proteins obtained unique regulatory characteristics. It is also interesting to note that all well-characterized barbed end capping proteins are composed of two homologous (or identical) actin binding domains (McGough et al., 2003; Wear and Cooper, 2004; Kovar, 2006). In conclusion, this study shows that the two twinfilin domains are structurally and biochemically different from each other, and evolved to play distinct roles during actin filament barbed end capping.

5. CONCLUDING REMARKS

The actin cytoskeleton is controlled by a large array of actin regulating proteins. In this work, we studied one of the highly conserved proteins, twinfilin, which is composed of two actin-binding domains, Twf-N and Twf-C. Previously it was known that twinfilin binds to actin monomers and to heterodimeric capping protein (CP), but the mechanisms of these interactions had remained unclear. Our studies revealed that both isolated domains bind to ADP-G-actin, but that Twf-C forms the high-affinity G-actin binding domain in full-length twinfilin. Further, we showed that Twf-N and Twf-C are structurally and biochemically different. Isolated Twf-C is structurally very similar to ADF/cofilin. It also binds F-actin and promotes pointed end disassembly of actin filaments, similarly to ADF/cofilin proteins. Twf-N has a different orientation of the β-protrusion that in ADF/cofilin is important for F-actin binding. This difference accounts for the inability of Twf-N to bind F-actin.

We identified the C-terminal tail region of twinfilin as the site of interaction with CP, and showed that this interaction in yeast cells is critical for twinfilin's correct localization to the dynamic actin patches. The *in vivo* importance of the twinfilin - CP -interaction, and the apparent lack of biochemical effects of this interaction suggest that in cells the interplay between these proteins is probably more complex than previously thought. It is possible that twinfilin and capping protein function synergistically in regulating the dynamic actin cytoskeleton (Figure 8). Also other, yet unidentified proteins might be involved in actin regulation by twinfilin and CP.

The presence of two active twinfilin domains is necessary for the capping activity of twinfilin, and Twf-C plays a critical role in this process. Our studies revealed that Twf-C binds to the sides of actin filaments with the help of the C-terminal tail region, and that its F-actin binding activity is critical to the capping activity of full-length twinfilin. We conclude that Twf-N binds only to the very terminal actin monomer and that the lower actin binding affinity of Twf-N probably acts in regulating the barbed end capping affinity of twinfilin (Figure 8). Furthermore, we suggest that twinfilin and gelsolin proteins evolved independently, to obtain a similar filament barbed end capping activity, from ancient ADF/cofilin and gelsolin -domain proteins, respectively (Figure 7B).

Taken together, this work revealed the specific roles of Twf-N and Twf-C during the interaction of twinfilin with actin monomers and filament barbed ends. The C-terminal tail region of twinfilin was shown to be important for filament side binding of Twf-C, and CP binding. In the future, it will be important to investigate how these two interactions participate in actin regulation. It will be important (although difficult) to reveal whether the twinfilin/actin monomer - complex can also bind to F-actin bound capping protein (Figure 8). Twinfilin, unlike CP, preferably caps the barbed ends of ADP-actin filaments. It is known that actin filament capping is necessary for generating a large number of very short filaments that are suitable for producing pushing forces for example on the plasma membrane or vesicles. The requirement for both domains in the capping activity of twinfilin provides an explanation for why twinfilin possesses the two actin binding domains. This and the fact that most actin monomers in cells are believed to exist

in their ATP-bound state (Rosenblatt et al., 1995) prompts the question whether the main cellular function of twinfilin is to cap ADP-actin filament barbed ends. This question could be answered with specific mutations, abolishing one of the three known activities of twinfilin at a time. Recently it has also been shown that the dynactin complex also contains CP (Eckley et al., 1999). Dynactin is a multi-protein complex, which links the dynein motor to cargo in vesicle transport along microtubules in the cytosol. Therefore, it is also possible that twinfilin also binds capping protein in its dynactin-bound form, and might have a role in connecting the actin- and microtubule -systems in cells.

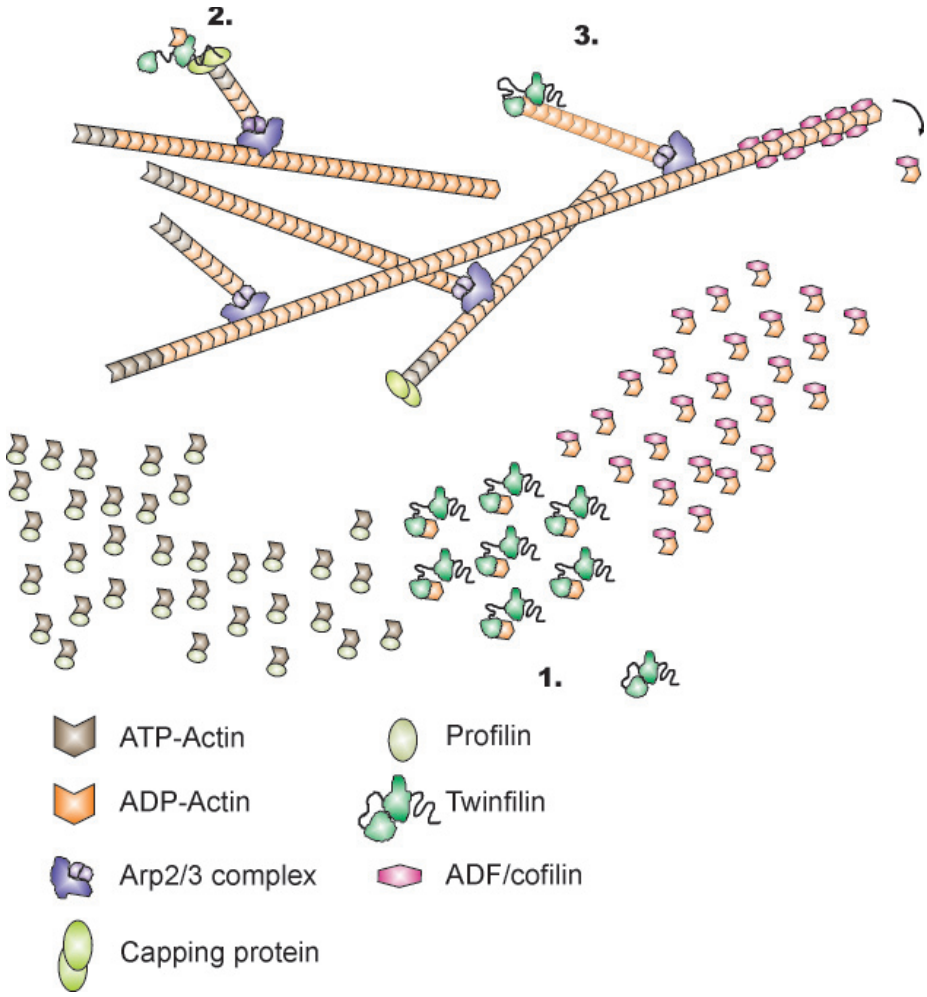


Fig 8. A schematic drawing describing the three possible roles of twinfilin in the dendritic nucleation model. Twinfilin can **1)** participate in the regulation of the size, localization and dynamics of the unpolymerized actin pool, **2)** act synergistically with capping protein to regulate actin filament assembly at the filament barbed ends, and **3)** directly associate with the filament barbed ends to prevent filament assembly.

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7. REFERENCES

Alberts Bruce, Bray Dennis, Lewis Julian, Raff Martin, Roberts Keith, and Watson James D. Molecular biology of the cell. 1994.

Andrianantoandro,E. and Pollard,T.D. (2006). Mechanism of actin filament turnover by severing and nucleation at different concentrations of ADF/cofilin. Mol. Cell. *24*, 13-23.

Anton,I.M., de la Fuente,M.A., Sims,T. N., Freeman,S., Ramesh,N., Hartwig,J.H., Dustin,M.L., and Geha,R.S. (2002). WIP deficiency reveals a differential role for WIP and the actin cytoskeleton in T and B cell activation. Immunity. *16*, 193-204.

Anton,I.M. and Jones,G.E. (2006). WIP: a multifunctional protein involved in actin cytoskeleton regulation. Eur. J. Cell Biol. *85*, 295-304.

Archer,S.K., Claudianos,C., and Campbell,H. D. (2005). Evolution of the gelsolin family of actin-binding proteins as novel transcriptional coactivators. Bioessays. *27*, 388-396.

Aspenstrom,P. (2005). The verprolin family of proteins: regulators of cell morphogenesis and endocytosis. FEBS Lett. *579*, 5253-5259.

Balcer,H.I., Goodman,A.L., Rodal,A.A., Smith,E., Kugler,J., Heuser,J.E., and Goode,B. L. (2003). Coordinated regulation of actin filament turnover by a high-molecular-weight Srv2/CAP complex, cofilin, profilin, and Aip1. Curr. Biol. *13*, 2159-2169.

Bamburg,J.R. (1999). Proteins of the ADF/ cofilin family: essential regulators of actin dynamics. Annu. Rev. Cell Dev. Biol. *15*, 185-230.

Baum,B., Li,W., and Perrimon,N. (2000). A cyclase-associated protein regulates actin and cell polarity during Drosophila oogenesis and in yeast. Curr. Biol. *10*, 964-973.

Bear,J.E., Svitkina,T.M., Krause,M., Schafer,D. A., Loureiro,J.J., Strasser,G.A., Maly,I.V., Chaga,O.Y., Cooper,J.A., Borisy,G.G., and Gertler,F.B. (2002). Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility. Cell. *109*, 509-521.

Benlali,A., Draskovic,I., Hazelett,D.J., and Treisman,J.E. (2000). act up controls actin polymerization to alter cell shape and restrict Hedgehog signaling in the Drosophila eye disc. Cell. *101*, 271-281.

Bertling,E., Hotulainen,P., Mattila,P.K., Matilainen,T., Salminen,M., and Lappalainen,P. (2004). Cyclase-associated-protein 1 (CAP1) promotes cofilin-induced actin dynamics in mammalian non-muscle cells. Mol. Biol. Cell.

Blanchoin,L. and Pollard,T.D. (1999). Mechanism of interaction of Acanthamoeba actophorin (ADF/Cofilin) with actin filaments. J. Biol. Chem. *274*, 15538-15546.

Blessing,C.A., Ugrinova,G.T., and Goodson,H. V. (2004). Actin and ARPs: action in the nucleus. Trends Cell Biol. *14*, 435-442.

Bowman,G.D., Nodelman,I.M., Hong,Y., Chua,N.H., Lindberg,U., and Schutt,C.E. (2000). A comparative structural analysis of the ADF/cofilin family. Proteins *41*, 374-384.

Brakebusch,C. and Fassler,R. (2003). The integrin-actin connection, an eternal love affair. EMBO J. *22*, 2324-2333.

Bruck,S., Huber,T.B., Ingham,R.J., Kim,K., Niederstrasser,H., Allen,P.M., Pawson,T., Cooper,J.A., and Shaw,A.S. (2006). Identification of a novel inhibitory actin-capping protein binding motif in CD2-associated protein. J. Biol. Chem. *281*, 19196-19203.

Burtnick,L.D., Urosev,D., Irobi,E., Narayan,K., and Robinson,R.C. (2004). Structure of the N-terminal half of gelsolin bound to actin: roles in severing, apoptosis and FAF. EMBO J. *23*, 2713-2722.

Canton,D.A., Olsten,M.E., Niederstrasser,H., Cooper,J.A., and Litchfield,D.W. (2006). The role of CKIP-1 in cell morphology depends on its interaction with actin-capping protein. J. Biol. Chem.

Carlier,M.F., Jean,C., Rieger,K.J., Lenfant,M., and Pantaloni,D. (1993). Modulation of the interaction between G-actin and thymosin beta 4 by the ATP/ADP ratio: possible implication in the regulation of actin dynamics. Proc. Natl. Acad. Sci. U. S. A *90*, 5034-5038.

Carlier,M.F., Laurent,V., Santolini,J., Melki,R., Didry,D., Xia,G.X., Hong,Y., Chua,N.H., and Pantaloni,D. (1997). Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: implication in actin-based motility. J. Cell Biol. *136*, 1307-1322.

Choe,H., Burtnick,L.D., Mejillano,M., Yin,H. L., Robinson,R.C., and Choe,S. (2002). The calcium activation of gelsolin: insights from the 3A structure of the G4-G6/actin complex. J. Mol. Biol. *324*, 691-702.

Cory,G.O., Cramer,R., Blanchoin,L., and Ridley,A.J. (2003). Phosphorylation of the WASP-VCA domain increases its affinity for the Arp2/3 complex and enhances actin polymerization by WASP. Mol. Cell. *11*, 1229-1239.

Craig,R., Greene,L.E., and Eisenberg,E. (1985). Structure of the actin-myosin complex in the presence of ATP. Proc. Natl. Acad. Sci. U. S. A. *82*, 3247-3251.

Croce,A., Cassata,G., Disanza,A., Gagliani,M. C., Tacchetti,C., Malabarba,M.G., Carlier,M. F., Scita,G., Baumeister,R., and Di Fiore,P. P. (2004). A novel actin barbed-end-capping activity in EPS-8 regulates apical morphogenesis in intestinal cells of Caenorhabditis elegans. Nat. Cell Biol. *6*, 1173-1179.

Dayel,M.J., Holleran,E.A., and Mullins,R.D. (2001). Arp2/3 complex requires hydrolyzable ATP for nucleation of new actin filaments. Proc. Natl. Acad. Sci. U. S. A. *98*, 14871-14876.

Disanza,A., Carlier,M.F., Stradal,T.E., Didry,D., Frittoli,E., Confalonieri,S., Croce,A., Wehland,J., Di Fiore,P.P., and Scita,G. (2004). Eps8 controls actin-based motility by capping the barbed ends of actin filaments. Nat. Cell Biol. *6*, 1180-1188.

Djinovic-Carugo,K., Gautel,M., Ylanne,J., and Young,P. (2002). The spectrin repeat: a structural platform for cytoskeletal protein assemblies. FEBS Lett. *%20;513*, 119-123.

Dodatko,T., Fedorov,A.A., Grynberg,M., Patskovsky,Y., Rozwarski,D.A., Jaroszewski,L., Aronoff-Spencer,E., Kondraskina,E., Irving,T., Godzik,A., and Almo,S.C. (2004). Crystal structure of the actin binding domain of the cyclase-associated protein. Biochemistry. *43*, 10628-10641.

Doi,M., Wachi,M., Ishino,F., Tomioka,S., Ito,M., Sakagami,Y., Suzuki,A., and Matsuhashi,M. (1988). Determinations of the DNA sequence of the mreB gene and of the gene products of the mre region that function in formation of the rod shape of Escherichia coli cells. J. Bacteriol. *170*, 4619-4624.

Dominguez,R. (2004). Actin-binding proteins--a unifying hypothesis. Trends Biochem. Sci. *29*, 572-578.

Eckley,D.M., Gill,S.R., Melkonian,K.A., Bingham,J.B., Goodson,H.V., Heuser,J.E., and Schroer,T.A. (1999). Analysis of dynactin subcomplexes reveals a novel actin-related protein associated with the arp1 minifilament pointed end. J. Cell Biol. *147*, 307-320.

Fedorov,A.A., Lappalainen,P., Fedorov,E.V., Drubin,D.G., and Almo,S.C. (1997). Structure determination of yeast cofilin. Nat. Struct. Biol. *4*, 366-369.

Fischer,R.S. and Fowler,V.M. (2003). Tropomodulins: life at the slow end. Trends Cell Biol. *13*, 593-601.

Fowler,V.M., Greenfield,N.J., and Moyer,J. (2003). Tropomodulin contains two actin filament pointed end-capping domains. J. Biol. Chem. *278*, 40000-40009.

Frischknecht,F., Moreau,V., Rottger,S., Gonfloni,S., Reckmann,I., Superti-Furga,G., and Way,M. (1999). Actin-based motility of vaccinia virus mimics receptor tyrosine kinase signalling. Nature. *401*, 926-929.

Galkin,V.E., Orlova,A., Lukoyanova,N., Wriggers,W., and Egelman,E.H. (2001). Actin depolymerizing factor stabilizes an existing state of F-actin and can change the tilt of F-actin subunits. J. Cell Biol. *153*, 75-86.

Garner,E.C., Campbell,C.S., and Mullins,R. D. (2004). Dynamic instability in a DNA-segregating prokaryotic actin homolog. Science. *306*, 1021-1025.

Gautreau,A., Ho,H.Y., Li,J., Steen,H., Gygi,S. P., and Kirschner,M.W. (2004). Purification and architecture of the ubiquitous Wave complex. Proc. Natl. Acad. Sci. U. S. A. *101*, 4379-4383.

Gill,S.R., Schroer,T.A., Szilak,I., Steuer,E. R., Sheetz,M.P., and Cleveland,D.W. (1991). Dynactin, a conserved, ubiquitously expressed component of an activator of vesicle motility mediated by cytoplasmic dynein. J. Cell Biol. *115*, 1639-1650.

Gohla,A., Birkenfeld,J., and Bokoch,G. M. (2005). Chronophin, a novel HAD-type serine protein phosphatase, regulates cofilin-dependent actin dynamics. Nat. Cell Biol. *7*, 21-29.

Goley,E.D. and Welch,M.D. (2006). The ARP2/3 complex: an actin nucleator comes of age. Nat. Rev. Mol. Cell Biol. *7*, 713-726.

Goode,B.L., Drubin,D.G., and Lappalainen,P. (1998). Regulation of the cortical actin cytoskeleton in budding yeast by twinfilin, a ubiquitous actin monomer-sequestering protein. J Cell Biol *142*, 723-733.

Gunning,P.W., Schevzov,G., Kee,A.J., and Hardeman,E.C. (2005). Tropomyosin isoforms: divining rods for actin cytoskeleton function. Trends Cell Biol. *15*, 333-341.

Hatanaka,H., Ogura,K., Moriyama,K., Ichikawa,S., Yahara,I., and Inagaki,F. (1996). Tertiary structure of destrin and structural similarity between two actin-regulating protein families. Cell *85*, 1047-1055.

Hawkins,M., Pope,B., Maciver,S.K., and Weeds,A.G. (1993). Human actin depolymerizing factor mediates a pH-sensitive destruction of actin filaments. Biochemistry *32*, 9985-9993.

Helfer,E., Nevalainen,E.M., Naumanen,P., Romero,S., Didry,D., Pantaloni,D., Lappalainen,P., and Carlier,M.F. (2006). Mammalian twinfilin sequesters ADP-G-actin and caps filament barbed ends: implications in motility. EMBO J. *25*, 1184-1195.

Hellman,M., Paavilainen,V.O., Naumanen,P., Lappalainen,P., Annila,A., and Permi,P. (2004). Solution structure of coactosin reveals structural homology to ADF/cofilin family proteins. FEBS Lett. *576*, 91-96.

Hertzog,M., van Heijenoort,C., Didry,D., Gaudier,M., Coutant,J., Gigant,B., Didelot,G., Preat,T., Knossow,M., Guittet,E., and Carlier,M.F. (2004). The beta-thymosin/WH2 domain; structural basis for the switch from inhibition to promotion of actin assembly. Cell. *117*, 611-623.

Higgs,H.N. (2004). There goes the neighbourhood: Eps8 joins the barbed-end crowd. Nat. Cell Biol. *6*, 1147-1149.

Higgs,H.N. (2005). Formin proteins: a domain-based approach. Trends Biochem. Sci. *30*, 342-353.

Ho,H.Y., Rohatgi,R., Lebensohn,A.M., Le,M., Li,J., Gygi,S.P., and Kirschner,M.W. (2004). Toca-1 mediates Cdc42-dependent actin nucleation by activating the N-WASP-WIP complex. Cell. *118*, 203-216.

Ho,H.Y., Rohatgi,R., Ma,L., and Kirschner,M. W. (2001). CR16 forms a complex with N-WASP in brain and is a novel member of a conserved proline-rich actin-binding protein family. Proc. Natl. Acad. Sci. U. S. A. *98*, 11306-11311.

Holm,L. and Sander,C. (1998). Touring protein fold space with Dali/FSSP. Nucleic. Acids Res *26*, 316-319.

Holmes,K.C., Popp,D., Gebhard,W., and Kabsch,W. (1990). Atomic model of the actin filament. Nature *347*, 44-49.

Hotulainen,P., Paunola,E., Vartiainen,M.K., and Lappalainen,P. (2005). Actin-depolymerizing factor and cofilin-1 play overlapping roles in promoting rapid F-actin depolymerization in mammalian nonmuscle cells. Mol. Biol. Cell. *16*, 649-664.

Hubberstey,A.V. and Mottillo,E.P. (2002). Cyclase-associated proteins: CAPacity for linking signal transduction and actin polymerization. FASEB J. *16*, 487-499.

Kabsch,W., Mannherz,H.G.,Suck,D.,Pai,E. F.,Holmes,K.C. (1990). Atomic structure of the actin:DNase I complex. Nature Sep 6;347(6288):37-44.

Kabsch,W. and Holmes,K.C. (1995). The actin fold. FASEB J. *9*, 167-174.

Kaksonen,M., Toret,C.P., and Drubin,D.G. (2005). A modular design for the clathrin- and actin-mediated endocytosis machinery. Cell. *123*, 305-320.

Kim,K., Yamashita,A., Wear,M.A., Maeda,Y., and Cooper,J.A. (2004). Capping protein binding to actin in yeast: biochemical mechanism and physiological relevance. J. Cell Biol. *164*, 567-580.

Kinley,A.W., Weed,S.A., Weaver,A.M., Karginov,A.V., Bissonette,E., Cooper,J.A., and Parsons,J.T. (2003). Cortactin interacts with WIP in regulating Arp2/3 activation and membrane protrusion. Curr. Biol. *13*, 384-393.

Kothakota,S., Azuma,T., Reinhard,C., Klippel,A., Tang,J., Chu,K., McGarry,T.J., Kirschner,M.W., Koths,K., Kwiatkowski,D. J., and Williams,L.T. (1997). Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. Science. *278*, 294-298.

Kovar,D.R. (2006). Molecular details of formin-mediated actin assembly. Curr. Opin. Cell Biol. *18*, 11-17.

Kovar,D.R., Harris,E.S., Mahaffy,R., Higgs,H. N., and Pollard,T.D. (2006). Control of the assembly of ATP- and ADP-actin by formins and profilin. Cell. *124*, 423-435.

Kraulis,P.J. (1991). MOLSCRIPT: A Program to Produce Both Detailed and Schematic Plots of Protein Structures. Journal of applied crystallography *24*, 946-950.

Laemmli,U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Lappalainen,P., Fedorov,E.V., Fedorov,A.A., Almo,S.C., and Drubin,D.G. (1997). Essential functions and actin-binding surfaces of yeast cofilin revealed by systematic mutagenesis. EMBO J. *16*, 5520-5530.

Lappalainen,P., Kessels,M.M., Cope,M.J., and Drubin,D.G. (1998). The ADF homology (ADF-H) domain: a highly exploited actin-binding module. Mol. Biol. Cell *9*, 1951-1959.

Le Clainche,C., Pantaloni,D., and Carlier,M.F. (2003). ATP hydrolysis on actin-related protein 2/3 complex causes debranching of dendritic actin arrays. Proc. Natl. Acad. Sci. U. S. A. *100*, 6337-6342.

Leonard,S.A., Gittis,A.G., Petrella,E.C., Pollard,T.D., and Lattman,E.E. (1997). Crystal structure of the actin-binding protein actophorin from Acanthamoeba. Nat. Struct. Biol. *4*, 369-373.

Levy,E., Haltia,M., Fernandez-Madrid,I., Koivunen,O., Ghiso,J., Prelli,F., and Frangione,B. (1990). Mutation in gelsolin gene in Finnish hereditary amyloidosis. J. Exp. Med. *172*, 1865-1867.

Lin,S.Y., Raval,S., Zhang,Z., Deverill,M., Siminovitch,K.A., Branch,D.R., and Haimovich,B. (2004). The protein-tyrosine phosphatase SHP-1 regulates the phosphorylation of alpha-actinin. J. Biol. Chem. *279*, 25755-25764.

Liu,J., Kaksonen,M., Drubin,D.G., and Oster,G. (2006). Endocytic vesicle scission by lipid phase boundary forces. Proc. Natl. Acad. Sci. U. S. A. *103*, 10277-10282.

Loisel,T.P., Boujemaa,R., Pantaloni,D., and Carlier,M.F. (1999). Reconstitution of actin-based motility of Listeria and Shigella using pure proteins. Nature *401*, 613-616.

Lowe,J. and Amos,L.A. (1998). Crystal structure of the bacterial cell-division protein FtsZ. Nature. *391*, 203-206.

Machesky,L.M., Atkinson,S.J., Ampe,C., Vandekerckhove,J., and Pollard,T.D. (1994). Purification of a cortical complex containing two unconventional actins from Acanthamoeba by affinity chromatography on profilin-agarose. J. Cell Biol. *127*, 107-115.

Maciver,S.K. and Weeds,A.G. (1994). Actophorin preferentially binds monomeric ADP-actin over ATP-bound actin: consequences for cell locomotion. FEBS Lett. *347*, 251-256.

Margolin,W. (2005). Bacterial mitosis: actin in a new role at the origin. Curr. Biol. *15*, R259-R261.

Martin,A.C., Welch,M.D., and Drubin,D. G. (2006). Arp2/3 ATP hydrolysis-catalysed branch dissociation is critical for endocytic force generation. Nat. Cell Biol. *8*, 826-833.

Martinez-Quiles,N., Rohatgi,R., Anton,I. M., Medina,M., Saville,S.P., Miki,H., Yamaguchi,H., Takenawa,T., Hartwig,J. H., Geha,R.S., and Ramesh,N. (2001). WIP regulates N-WASP-mediated actin polymerization and filopodium formation. Nat. Cell Biol. *3*, 484-491.

Mattila,P.K., Quintero-Monzon,O., Kugler,J., Moseley,J.B., Almo,S.C., Lappalainen,P., and Goode,B.L. (2004). A high-affinity interaction with ADP-actin monomers underlies the mechanism and in vivo function of Srv2/cyclase-associated protein. Mol. Biol. Cell. *15*, 5158-5171.

Maury,C.P., Kere,J., Tolvanen,R., and de la,C. A. (1990). Finnish hereditary amyloidosis is caused by a single nucleotide substitution in the gelsolin gene. FEBS Lett. *276*, 75-77.

Mavoungou,C., Israel,L., Rehm,T., Ksiazek,D., Krajewski,M., Popowicz,G., Noegel,A.A., Schleicher,M., and Holak,T.A. (2004). NMR structural characterization of the N-terminal domain of the adenylyl cyclase-associated protein (CAP) from Dictyostelium discoideum. J. Biomol. NMR. *29*, 73-84.

McGough,A., Pope,B., Chiu,W., and Weeds,A. (1997). Cofilin changes the twist of F-actin: implications for actin filament dynamics and cellular function. J. Cell Biol. *138*, 771-781.

McGough,A.M., Staiger,C.J., Min,J.K., and Simonetti,K.D. (2003). The gelsolin family of actin regulatory proteins: modular structures, versatile functions. FEBS Lett. *552*, 75-81.

McLaughlin,P.J., Gooch,J.T., Mannherz,H.G., and Weeds,A.G. (1993). Structure of gelsolin segment 1-actin complex and the mechanism of filament severing. Nature. *19;364*, 685-692.

Merrit,E.A., Murphy,M.E.P., and . (1994). Raster3D Version 2.0: A Program for Photorealistic Molecular Graphics. Acta Crystallographica Section D-Biological Crystallography *D50*, 873.

Moreau,V., Frischknecht,F., Reckmann,I., Vincentelli,R., Rabut,G., Stewart,D., and Way,M. (2000). A complex of N-WASP and WIP integrates signalling cascades that lead to actin polymerization. Nat. Cell Biol. *2*, 441-448.

Moriyama,K. and Yahara,I. (2002). Human CAP1 is a key factor in the recycling of cofilin and actin for rapid actin turnover. J. Cell Sci. *115*, 1591-1601.

Moseley,J.B., Okada,K., Balcer,H.I., Kovar,D. R., Pollard,T.D., and Goode,B.L. (2006). Twinfilin is an actin-filament-severing protein and promotes rapid turnover of actin structures in vivo. J. Cell Sci. *119*, 1547-1557.

Muller,J., Oma,Y., Vallar,L., Friederich,E., Poch,O., and Winsor,B. (2005). Sequence and comparative genomic analysis of actin-related proteins. Mol. Biol. Cell. *16*, 5736-5748.

Mullins,R.D., Heuser,J.A., and Pollard,T.D. (1998). The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. Proc. Natl. Acad. Sci. U. S. A. *95*, 6181-6186.

Newpher,T.M., Smith,R.P., Lemmon,V., and Lemmon,S.K. (2005). In vivo dynamics of clathrin and its adaptor-dependent recruitment to the actin-based endocytic machinery in yeast. Dev. Cell. *9*, 87-98.

Niwa,R., Nagata-Ohashi,K., Takeichi,M., Mizuno,K., and Uemura,T. (2002). Control of actin reorganization by Slingshot, a family of phosphatases that dephosphorylate ADF/cofilin. Cell *108*, 233-246.

Noegel,A.A., Blau-Wasser,R., Sultana,H., Muller,R., Israel,L., Schleicher,M., Patel,H., and Weijer,C.J. (2004). The cyclase-associated protein CAP as regulator of cell polarity and cAMP signaling in Dictyostelium. Mol. Biol. Cell. *15*, 934-945.

Nolen,B.J., Littlefield,R.S., and Pollard,T. D. (2004). Crystal structures of actin-related protein 2/3 complex with bound ATP or ADP. Proc. Natl. Acad. Sci. U. S. A. *101*, 15627-15632.

Oikawa,T., Yamaguchi,H., Itoh,T., Kato,M., Ijuin,T., Yamazaki,D., Suetsugu,S., and Takenawa,T. (2004). PtdIns(3,4,5)P3 binding is necessary for WAVE2-induced formation of lamellipodia. Nat. Cell Biol. *6*, 420-426.

Okada,K., Blanchoin,L., Abe,H., Chen,H., Pollard,T.D., and Bamburg,J.R. (2002). Xenopus actin-interacting protein 1 (XAip1) enhances cofilin fragmentation of filaments by capping filament ends. J. Biol. Chem. *277*, 43011-43016.

Ono,S. (2003). Regulation of actin filament dynamics by actin depolymerizing factor/cofilin and actin-interacting protein 1: new blades for twisted filaments. Biochemistry. *42*, 13363-13370.

Otey,C.A. and Carpen,O. (2004). Alpha-actinin revisited: a fresh look at an old player. Cell Motil. Cytoskeleton. *58*, 104-111.

Otomo,T., Tomchick,D.R., Otomo,C., Panchal,S.C., Machius,M., and Rosen,M. K. (2005). Structural basis of actin filament nucleation and processive capping by a formin homology 2 domain. Nature. *433*, 488-494.

Otterbein,L.R., Cosio,C., Graceffa,P., and Dominguez,R. (2002). Crystal structures of the vitamin D-binding protein and its complex with actin: structural basis of the actin-scavenger system. Proc. Natl. Acad. Sci. U. S. A. *99*, 8003-8008.

Otterbein,L.R., Grafecca,P., and Dominguez,R. (2001). The crystal structure of uncomplexed actin in the ADP state. Science. 2001 Jul 27;293(5530):708-11.

Paavilainen,V.O., Bertling,E., Falck,S., and Lappalainen,P. (2004). Regulation of cytoskeletal dynamics by actin-monomer-binding proteins. Trends Cell Biol. *14*, 386-394.

Palmgren,S., Ojala,P.J., Wear,M.A., Cooper,J. A., and Lappalainen,P. (2001). Interactions with PIP2, ADP-actin monomers, and capping protein regulate the activity and localization of yeast twinfilin. J. Cell Biol. *155*, 251-260.

Pelkmans,L., Fava,E., Grabner,H., Hannus,M., Habermann,B., Krausz,E., and Zerial,M. (2005). Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. Nature. *436*, 78-86.

Pollard,T.D., Blanchoin,L., and Mullins,R.D. (2000). Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. Annu Rev Biophys Biomol Struct *29*, 545-576.

Pollard,T.D. and Borisy,G.G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. Cell *112*, 453-465.

Prehoda,K.E., Scott,J.A., Mullins,R.D., and Lim,W.A. (2000). Integration of multiple signals through cooperative regulation of the N-WASP-Arp2/3 complex. Science. *290*, 801-806.

Pudas,R., Kiema,T.R., Butler,P.J., Stewart,M., and Ylanne,J. (2005). Structural basis for vertebrate filamin dimerization. Structure. *13*, 111-119.

Quinlan,M.E., Heuser,J.E., Kerkhoff,E., and Mullins,R.D. (2005). Drosophila Spire is an actin nucleation factor. Nature. *433*, 382-388.

Quintero-Monzon,O., Rodal,A.A., Strokopytov,B., Almo,S.C., and Goode,B.L. (2005). Structural and functional dissection of the Abp1 ADFH actin-binding domain reveals versatile in vivo adapter functions. Mol. Biol. Cell. *16*, 3128-3139.

Rajfur,Z., Roy,P., Otey,C., Romer,L.H., and Jacobson,K.A. (2001). The EGFP molecule mediates Chromophore Assisted Laser Inactivation of &similar to alpha-actinin. Biophysical Journal *80*, 277A-278A.

Ressad,F., Didry,D., Xia,G.X., Hong,Y., Chua,N.H., Pantaloni,D., and Carlier,M.F. (1998). Kinetic analysis of the interaction of actin-depolymerizing factor (ADF)/cofilin with G- and F-actins. Comparison of plant and human ADFs and effect of phosphorylation. J. Biol. Chem. *273*, 20894-20902.

Robinson,R.C., Turbedsky,K., Kaiser,D. A., Marchand,J.B., Higgs,H.N., Choe,S., and Pollard,T.D. (2001). Crystal structure of Arp2/3 complex. Science. *294*, 1679-1684.

Roeben,A., Kofler,C., Nagy,I., Nickell,S., Hartl,F.U., and Bracher,A. (2006). Crystal structure of an archaeal actin homolog. J. Mol. Biol. *358*, 145-156.

Rogers,S.L., Wiedemann,U., Stuurman,N., and Vale,R.D. (2003). Molecular requirements for actin-based lamella formation in Drosophila S2 cells. J. Cell Biol. *162*, 1079-1088.

Romero,S., Le Clainche,C., Didry,D., Egile,C., Pantaloni,D., and Carlier,M.F. (2004). Formin is a processive motor that requires profilin to accelerate actin assembly and associated ATP hydrolysis. Cell. *119*, 419-429.

Rosales-Nieves,A.E., Johndrow,J.E., Keller,L. C., Magie,C.R., Pinto-Santini,D.M., and Parkhurst,S.M. (2006). Coordination of microtubule and microfilament dynamics by Drosophila Rho1, Spire and Cappuccino. Nat. Cell Biol. *8*, 367-376.

Rosenblatt,J., Peluso,P., and Mitchison,T. J. (1995). The bulk of unpolymerized actin in Xenopus egg extracts is ATP-bound. Mol. Biol. Cell. *6*, 227-236.

Safer,D. (1989). An electrophoretic procedure for detecting proteins that bind actin monomers. Anal. Biochem. *178*, 32-37.

Salazar,M.A., Kwiatkowski,A.V., Pellegrini,L., Cestra,G., Butler,M.H., Rossman,K.L., Serna,D.M., Sondek,J., Gertler,F.B., and De Camilli,P. (2003). Tuba, a novel protein containing bin/amphiphysin/Rvs and Dbl homology domains, links dynamin to regulation of the actin cytoskeleton. J. Biol. Chem. *278*, 49031-49043.

Schafer,D.A. and Schroer,T.A. (1999). Actin-related proteins. Annu. Rev. Cell Dev. Biol. *15:341-63.*, 341-363.

Schutt,C.E., Myslik,J.C., Rozycki,M.D., Goonesekere,N.C., and Lindberg,U. (1993). The structure of crystalline profilin-beta-actin. Nature *365*, 810-816.

Sheterline,P., Clayton,J., and Sparrow,J. (1995). Actin. Protein Profile. *2*, 1-103.

Soosairajah,J., Maiti,S., Wiggan,O., Sarmiere,P., Moussi,N., Sarcevic,B., Sampath,R., Bamburg,J.R., and Bernard,O. (2005). Interplay between components of a novel LIM kinase-slingshot phosphatase complex regulates cofilin. EMBO J. *24*, 473-486.

Spudich,J.A. and Watt,S. (1971). The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. J. Biol. Chem. *246*, 4866-4871.

Steffen,A., Rottner,K., Ehinger,J., Innocenti,M., Scita,G., Wehland,J., and Stradal,T.E. (2004). Sra-1 and Nap1 link Rac to actin assembly driving lamellipodia formation. EMBO J. *23*, 749-759.

Stradal,T.E., Rottner,K., Disanza,A., Confalonieri,S., Innocenti,M., and Scita,G. (2004). Regulation of actin dynamics by WASP and WAVE family proteins. Trends Cell Biol. *14*, 303-311.

Stradal,T.E. and Scita,G. (2006). Protein complexes regulating Arp2/3-mediated actin assembly. Curr. Opin. Cell Biol. *18*, 4-10.

Svergun,D.I., Petoukhov,M.V., and Koch,M. H. (2001). Determination of domain structure of proteins from X-ray solution scattering. Biophys. J. *80*, 2946-2953.

Taoka,M., Ichimura,T., Wakamiya-Tsuruta,A., Kubota,Y., Araki,T., Obinata,T., and Isobe,T. (2003). V-1, a protein expressed transiently during murine cerebellar development, regulates actin polymerization via interaction with capping protein. J. Biol. Chem. *278*, 5864-5870.

Tilney,L.G. and DeRosier,D.J. (2005). How to make a curved Drosophila bristle using straight actin bundles. Proc. Natl. Acad. Sci. U. S. A. *102*, 18785-18792.

Uruno,T., Remmert,K., and Hammer,J.A., III (2006). CARMIL is a potent capping protein antagonist: identification of a conserved

CARMIL domain that inhibits the activity of capping protein and uncaps capped actin filaments. J. Biol. Chem. *281*, 10635-10650.

van den Ent,F., Amos,L.A., and Lowe,J. (2001). Prokaryotic origin of the actin cytoskeleton. Nature. *413*, 39-44.

van den Ent,F. and Lowe,J. (2000). Crystal structure of the cell division protein FtsA from Thermotoga maritima. EMBO J. *19*, 5300-5307.

van den Ent,F., Moller-Jensen,J., Amos,L. A., Gerdes,K., and Lowe,J. (2002). F-actin-like filaments formed by plasmid segregation protein ParM. EMBO J. *21*, 6935-6943.

Vartiainen,M., Ojala,P.J., Auvinen,P., Peranen,J., and Lappalainen,P. (2000). Mouse A6/twinfilin is an actin monomer-binding protein that localizes to the regions of rapid actin dynamics. Mol. Cell Biol. *20*, 1772-1783.

Vartiainen,M.K., Sarkkinen,E.M., Matilainen,T., Salminen,M., and Lappalainen,P. (2003). Mammals have two twinfilin isoforms whose subcellular localizations and tissue distributions are differentially regulated. J. Biol. Chem. *278*, 34347-34355.

Vinson,V.K., De La Cruz,E.M., Higgs,H. N., and Pollard,T.D. (1998). Interactions of Acanthamoeba profilin with actin and nucleotides bound to actin. Biochemistry. *37*, 10871-10880.

Virel,A. and Backman,L. (2004). Molecular evolution and structure of alpha-actinin. Mol. Biol. Evol. *21*, 1024-1031.

Volkmann,N., Amann,K.J., Stoilova-McPhie,S., Egile,C., Winter,D.C., Hazelwood,L., Heuser,J.E., Li,R., Pollard,T. D., and Hanein,D. (2001). Structure of Arp2/3 complex in its activated state and in actin filament branch junctions. Science. *293*, 2456-2459.

Wahlstrom,G., Vartiainen,M., Yamamoto,L., Mattila,P.K., Lappalainen,P., and Heino,T.I. (2001). Twinfilin is required for actin-dependent developmental processes in Drosophila. J. Cell Biol. *155*, 787-796.

Wear,M.A. and Cooper,J.A. (2004). Capping protein: new insights into mechanism and regulation. Trends Biochem. Sci. *29*, 418-428.

Weaver,A.M., Karginov,A.V., Kinley,A.W., Weed,S.A., Li,Y., Parsons,J.T., and Cooper,J. A. (2001). Cortactin promotes and stabilizes Arp2/3-induced actin filament network formation. Curr. Biol. *11*, 370-374.

Weber,A., Pennise,C.R., Babcock,G.G., and Fowler,V.M. (1994). Tropomodulin caps the pointed ends of actin filaments. J. Cell Biol. *127*, 1627-1635.

Weber,A., Pennise,C.R., and Fowler,V.M. (1999). Tropomodulin increases the critical concentration of barbed end-capped actin filaments by converting ADP.P(i)-actin to ADP-actin at all pointed filament ends. J. Biol. Chem. *274*, 34637-34645.

Welch,M.D., Iwamatsu,A., and Mitchison,T. J. (1997). Actin polymerization is induced by Arp2/3 protein complex at the surface of Listeria monocytogenes. Nature. *385*, 265-269.

Wiesner,S., Helfer,E., Didry,D., Ducouret,G., Lafuma,F., Carlier,M.F., and Pantaloni,D. (2003). A biomimetic motility assay provides insight into the mechanism of actin-based motility. J. Cell Biol. *160*, 387-398.

Witke,W. (2004). The role of profilin complexes in cell motility and other cellular processes. Trends Cell Biol. *14*, 461-469.

Wriggers,W., Tang,J.X., Azuma,T., Marks,P. W., and Janmey,P.A. (1998). Cofilin and gelsolin segment-1: molecular dynamics simulation and biochemical analysis predict a similar actin binding mode. J. Mol. Biol. *282*, 921-932.

Xu,Y., Moseley,J.B., Sagot,I., Poy,F., Pellman,D., Goode,B.L., and Eck,M.J. (2004). Crystal structures of a Formin Homology-2 domain reveal a tethered dimer architecture. Cell. *116*, 711-723.

Yamashita,A., Maeda,K., and Maeda,Y. (2003). Crystal structure of CapZ: structural basis for actin filament barbed end capping. EMBO J. *22*, 1529-1538.

Yarmola,E.G. and Bubb,M.R. (2006). Profilin: emerging concepts and lingering misconceptions. Trends Biochem. Sci. *31*, 197-205.

Yonezawa,N.,Nishida,E.,Iida,K.,Yahara,I.,and Sakai,H. (1990). Inhibition of the interactions of cofilin, destrin, and deoxyribonuclease I with actin by phosphoinositides. J. Biol. Chem. *265*, 8382-8386.

Zhang,Z., Lin,S.Y., Neel,B.G., and Haimovich,B. (2006). Phosphorylated alpha-actinin and protein-tyrosine phosphatase 1B coregulate the disassembly of the focal adhesion kinase x Src complex and promote cell migration. J. Biol. Chem. *281*, 1746-1754.